

Birth Defects Research (Part C): Embryo Today

Reviews

REGENERATION IN DEVELOPMENT

September 2013

Osteoblast Recruitment to Sites of Bone Formation in Skeletal Development, Homeostasis and Regeneration

Naomi Dirckx, Matthias Van Hul, and Christa Maes*

All authors are from the Laboratory of Skeletal Cell Biology and Physiology (SCEBP), Skeletal Biology and Engineering Research Center (SBE), Department of Development and Regeneration, KU Leuven, Leuven, Belgium

Grant sponsor: CM received funding from the European Research Council (ERC, Starting Grant 282131) under the European Union's Seventh Framework Programme (FP7/2007-2013); MVH is a postdoctoral fellow of the Fund for Scientific Research of Flanders (FWO).

***Correspondence to:**

Christa Maes

Laboratory of Skeletal Cell Biology and Physiology (SCEBP)

Skeletal Biology and Engineering Research Center (SBE)

Gasthuisberg O&N 1, box 813.

Herestraat 49, B-3000 Leuven, Belgium.

Tel: +32-16-346183.

E-mail: christa.maes@med.kuleuven.be

Osteoblast Recruitment to Sites of Bone Formation in Skeletal Development, Homeostasis and Regeneration - Review outline:

ABSTRACT

INTRODUCTION

OSTEOBLAST ORIGIN AND RECRUITMENT INTO DEVELOPING LONG BONES

- Ossification in skeletal development
- Osteoblast differentiation
- Origin of osteoblasts in endochondral bone development: the perichondrium as source of osteoprogenitors for trabecular bone formation

CONTROL OF OSTEOBLAST RECRUITMENT AND BONE FORMATION DURING DEVELOPMENT AND REMODELING BY CHEMOTACTIC AND OSTEO-ANGIOGENIC COUPLING FACTORS

- Growth factors inducing chemotaxis of osteoblast lineage cells in vitro
- Osteoblast recruitment during bone remodeling in vivo
- Bone formation is coupled to vascularization: regulation by hypoxia signaling and VEGF
 - *Regulation of bone vascularization by VEGF: lessons from genetically modified mouse models*
 - *HIF pathway involvement in osteo-angiogenic coupling*

RECAPITULATION OF EMBRYONIC MECHANISMS DURING ENDOCHONDRAL BONE REPAIR: OSTEOBLAST RECRUITMENT COUPLED TO NEOVASCULARIZATION

PERSPECTIVES

ACKNOWLEDGEMENTS

ABSTRACT

During endochondral bone development, bone-forming osteoblasts have to colonize the regions of cartilage that will be replaced by bone. In adulthood, bone remodeling and repair require osteogenic cells to reach the sites that need to be rebuilt, as a prerequisite for skeletal health. A failure of osteoblasts to reach the sites in need of bone formation may contribute to impaired fracture repair. Conversely, stimulation of osteoblast recruitment may be a promising osteo-anabolic strategy to improve bone formation in low bone mass disorders such as osteoporosis and in bone regeneration applications. Yet, still relatively little is known about the cellular and molecular mechanisms controlling osteogenic cell recruitment to sites of bone formation. In vitro, several secreted growth factors induce osteogenic cell migration. Recent studies started to shed light on the role of such chemotactic signals in osteoblast recruitment during bone remodeling. Trafficking of osteoblast lineage cells into new bone centers during endochondral bone development and repair is largely confined to undifferentiated osteoprogenitors, and coupled to angiogenic invasion of the cartilage intermediate. It is well known that blood vessels are absolutely required for bone formation and that a close spatial-temporal relationship exists between osteogenesis and angiogenesis. Studies using genetically modified mice have identified some molecular constituents of this osteogenic-angiogenic coupling. This article reviews the current knowledge on osteoblast recruitment to sites of active bone formation in skeletal development, remodeling, and repair, considering the role of chemo-attractants for osteogenic cells and the interplay between osteogenesis and angiogenesis in the control of bone formation.

INTRODUCTION

One of the major goals in the bone field today is to identify new approaches to stimulate bone formation. Clinically, anabolic drugs are much needed to help restore the bone mass in osteoporotic patients and to improve repair of large bone defects.

Osteoporosis, the disease of ‘porous bones’, is very widespread in older people and imposes a huge public health problem. Normal bone is remodeled throughout adult life, undergoing continual renewal and adaptation to physical exercise, diet and other factors. This is mediated by the balanced activities of bone-forming osteoblasts and bone-resorbing osteoclasts. Osteoporosis results from a dysregulation of this finely concerted balance, with osteoclastic bone resorption exceeding osteoblastic bone formation, leading to net bone loss. Current therapies effectively halt bone destruction, but are unable to reverse the disease; there is a large need for additional anabolic drugs that stimulate bone formation and improve bone strength beyond what can be achieved with antiresorptives (Baron and Hesse, 2012, Rachner et al., 2011).

Key to the development of new anabolic bone therapies is a full understanding of the molecular mechanisms that accommodate the functioning of bone-producing osteoblasts. Osteoblast differentiation, from mesenchymal precursors that first commit to the lineage as osteoprogenitors, is therefore intensively studied. One aspect of bone formation that received relatively less attention is the recruitment of osteogenic cells to the active bone surfaces. Yet, mature osteoblasts are located onto the bone surfaces, whereas osteoprogenitors reside at the periphery of the bone shaft or dispersed in the bone environment. The recruitment of osteoprogenitors to the bone surface is therefore an indispensable aspect of bone formation; and, modulating this process could offer unique opportunities for anabolic treatment of osteoporosis and other metabolic bone disorders. Likewise, increased attraction of osteoblasts to sites in need of bone formation could be a promising strategy to treat compromised fracture repair and improve (tissue-engineered) bone regeneration. Fracture repair in the adult closely resembles bone development and recapitulates several of the main molecular pathways operating in fetal life (Gerstenfeld et al., 2003, Schindeler et al., 2008). A thorough understanding of the mechanisms governing embryonic development and normal physiology will thus add valuable insights in disease and regeneration, and be the basis for new therapeutic developments in the future.

Despite the great variety of shapes and sizes, bones develop and grow through a small number of cellular mechanisms. Endochondral bone formation is the most common strategy, forming all the long bones of the axial (vertebrae, ribs) and appendicular skeleton (limbs). This term indicates that mineralized bones form by organizing the cells and matrix of bone on scaffolding cartilage models. Two absolute requirements for normal endochondral bone formation are, firstly, the provision of bone-forming osteoblasts and secondly, progressive neovascularization of the growing bone. These two aspects go hand in hand, during development as well as in healing bone fractures. In fact, bone formation in virtually all settings occurs in close spatial and temporal association with vascularization of the ossified tissue, a concept termed angiogenic-osteogenic coupling (Schipani et al., 2009, Maes, 2013, Riddle et al., 2009). The interplay is dynamic and reciprocal, in that the cells of bone on the one hand depend on the signals, oxygen, and nutrient delivery via the blood stream, while on the other hand they influence the vascular arrangement and function. Moreover, some osteogenic cells with the capacity to become functional osteoblasts reside in the blood vessel wall. Altogether, the current knowledge suggests that osteoprogenitors and endothelial cells may be attracted to sites where vascularized bone is to be formed by common chemotactic stimuli and/or osteo-angiogenic coupling factors. The insights gained over the years through in vitro studies and a myriad of genetically modified animal models on these regulatory cues is reviewed here.

OSTEOBLAST ORIGIN AND RECRUITMENT INTO DEVELOPING LONG BONES

Ossification in skeletal development

The vertebrate skeleton is composed of over 200 individual bones, each with its own unique shape, size, location and function. The two major categories of bones are the flat bones, of which the skull is the most typical representative, and the long bones that comprise most bones in the skeleton, including those of the limbs (Figure 1). The flat bones consist of compact bone with a layer of interspersed bone marrow. The long bones characteristically consist of a shaft or diaphysis in the middle made up by dense cortical or compact bone, surrounding the medullary cavity, and epiphyses at both ends; the region in between is termed the metaphysis. The ends of the bone contain a honeycomb-like trabecular network of spongy bone on the inside. The cartilaginous growth plates, responsible for growth, are located

between the epiphysis and diaphysis, until they disappear ('close') at puberty in humans. The outer surface of the bone shaft is covered by a fibrous connective tissue termed periosteum, which is important for growth and fracture repair; the inner lining of the cortical bone of the diaphysis is termed the endosteum. At the epiphyses a layer of articular cartilage covers the bone, which is important for the functioning of the joints.

The flat bones of the skull and the long bones of the axial skeleton and the limbs develop through different mechanisms: intramembranous and endochondral ossification, respectively (Figure 1). Skeletal morphogenesis involves highly coordinated sequential steps of cell migration, aggregation, condensation, and organogenesis. Cells that originate from the cranial neural crest, the somites and the lateral plate mesoderm form the skeletal structures. Before intramembranous ossification can start, neural crest and paraxial mesodermal cells need to migrate in the right time frame to the right place for proper formation of the sutures (Helms and Schneider, 2003). This is followed by the condensation of mesenchymal cells that will form the skeletal structures, and their subsequent differentiation into osteoblasts. The osteoblasts deposit bone matrix rich in type I collagen (Col1) that next becomes mineralized. Endochondral ossification comprises of a more complicated process that gives rise to the long bones of the axial skeleton, derived from the paraxial mesoderm, and the appendicular skeleton, derived from lateral plate mesoderm (Shum et al., 2003). At sites where the bones will be formed, mesenchymal progenitor cells aggregate and form high-density condensations, representing the molds of the future skeletal elements. In mice, this process takes place around E10-E12. These condensed mesenchymal cells differentiate into chondrocytes, forming a scaffolding anlage of cartilage that is gradually replaced by bone during endochondral ossification. The mechanisms underlying the early migration, condensation, segmentation and differentiation events, define the precise arrangement of the individual anatomic elements and their patterning along the proximal-distal, dorsal-ventral, and anterior-posterior body axes. Excellent reviews are available on this subject (Tabin and Wolpert, 2007, Towers et al., 2012); here, we will focus on the later process of actual bone formation or ossification.

Ossification in developing long bones starts in the perichondrium, the connective tissue surrounding the cartilaginous template, where cells differentiate into osteoblasts that start to deposit and mineralize a structure called the 'bone collar' around the cartilage mold. This bone collar forms the initiation site of the cortical bone, the dense outer envelope of compact

bone that provides most of the strength and rigidity to the long bones. The actual process of ‘endochondral’ ossification is triggered shortly thereafter, around E14-E15 in mice, when the hypertrophic chondrocytes in the center of the cartilage mold become invaded by blood vessels along with osteoclasts and osteoprogenitors from the surrounding perichondrium (see further). As a result, the cartilage template is progressively eroded and replaced by trabecular bone and bone marrow, a region initially termed the primary ossification center. In the metaphysis, hypertrophic cartilage of the growth cartilage is continually replaced with trabecular bone, a process that relies heavily on angiogenesis and mediates longitudinal bone growth. After birth, the cartilage of the epiphysis also becomes invaded by blood vessels, emanating from the vascular network around the tissue. This gives rise to the secondary ossification center. The remaining cartilage between the ossification centers now forms a true growth plate ‘plate’, driving further longitudinal bone growth. In humans, the deposition of cartilage ceases at puberty; the metaphysis fuses with the epiphysis and growth stops. In mice, longitudinal growth slows dramatically at puberty, but the growth plates do not completely disappear.

Hence, in endochondral bones, osteoblast lineage cells will populate the region formerly occupied by chondrocytes, where they will differentiate and deposit osteoid on cartilage remnants. Understanding the process of their recruitment to the sites where bone will be formed may eventually help identify novel therapeutic targets to treat osteoporosis, improve impaired bone healing and contribute to the engineering of bone regeneration applications.

Osteoblast differentiation

Osteoblast lineage cells, encompassing osteoprogenitors, osteoblasts and osteocytes, derive from mesenchymal progenitor cells commonly referred to as mesenchymal stem cells (MSCs). MSCs from bone marrow, periosteum, and other sources are capable of differentiating along the osteoblastic, chondrogenic, adipogenic, and/or myogenic cell lineages (Augello et al., 2010, Tare et al., 2008). These differentiation processes are tightly regulated by transcription factors in a spatially and temporally controlled manner (Long, 2012, Hartmann, 2009) (Figure 2).

The commitment of mesenchymal progenitor cells into chondrogenic and osteogenic lineages starts with the expression of Sox9 (Akiyama et al., 2005). In bones that develop through endochondral ossification, a stage of bipotency of osteo-chondroprogenitors is recognized

before the cells specify in either the chondrocyte or the osteoblast lineage. Early osteoblast progenitors express Runx2, a Runt domain containing transcription factor that is absolutely required for the differentiation of osteoblasts as well as for the appropriate functioning of mature osteoblasts. Runx2 is viewed as the master controller of osteoblastogenesis. Its deletion in mice resulted in a cartilaginous skeleton with absence of osteogenic differentiation and bone formation (both intramembranous and endochondral) (Komori, 2010). A similar phenotype was observed in mice with inactivation of the Sp7 gene encoding Osterix (Osx), a second transcription factor expressed in osteoprogenitors that is essential for osteoblast differentiation and function (Nakashima et al., 2002). In situ hybridization studies using these knockout mouse models revealed that the expression of Runx2 was preserved in the perichondrium of endochondral bones as well as in the condensations of membranous skeletal elements of Osx null mice, whereas conversely, Osx expression was undetectable in the perichondrium surrounding the cartilage anlagen of mice lacking Runx2 (Nakashima et al., 2002). These findings established that Osx is genetically downstream of Runx2; yet, how much Runx2⁺ and Runx2⁺Osx⁺ osteogenic cell populations in vivo may or may not differ from each other in their molecular and functional characteristics remains to be clarified. Both Runx2 and Osx support the downstream effects of multiple osteogenic factors and regulate gene expression of many major bone matrix proteins during osteoblast differentiation. Runx2 also keeps a pool of osteoblasts in an immature stage and regulates chondrocyte differentiation (see below) (Long, 2012, Komori, 2010).

The third important transcription factor regulating osteoblast differentiation is β -catenin, the major mediator of canonical Wnt-signaling. β -catenin is indispensable for mesenchymal precursor cells to become Runx2⁺Osx⁺ and for the latter to differentiate into mature osteoblasts; deletion of the gene encoding β -catenin in Osx⁺ osteoprogenitors inhibited their terminal differentiation to mature osteoblasts, and instead induces the expression of chondrocytic or adipocytic markers (Rodda and McMahon, 2006, Song et al., 2012, Chen and Long, 2013, Hill et al., 2005, Case and Rubin, 2010).

As they mature, osteoblasts start producing abundant matrix proteins, including large quantities of the main bone constituent type I collagen, which they deposit as osteoid or non-mineralized bone matrix. This osteoid becomes mineralized by accumulation of calcium phosphate in the form of hydroxyapatite, a process executed by mature osteoblasts that reached a differentiation stage in which they typically express osteocalcin. Some of the cells ultimately undergo apoptosis or become bone lining cells, while another subset of osteoblasts

becomes embedded in the bone matrix and further differentiates to osteocytes (expressing dentin matrix protein 1 (Dmp1) and sclerostin). Osteocytes form a canalicular network of long protrusions to communicate with other osteocytes, and to osteoclasts and osteoblasts on the bone surface. They are highly mechano-sensitive cells and prime regulators of bone remodeling (Bonewald, 2011).

The line of progression of osteoblast differentiation is illustrated in Figure 2, with indication of some of the abovementioned markers typifying particular stages of the process. The promoters of several of these genes have been used to drive Cre expression in mice, constitutively and/or in temporal-regulated ways, to target genes of interest in genetically modified mice. The Cre-strains used most routinely in studies of osteoblast lineage cells include those driven by the *Osx* promoter, various versions of the *Col1* promoter, and the osteocalcin and *Dmp1* gene promoters (Figure 2) (Elefteriou and Yang, 2011).

Origin of osteoblasts in endochondral bone development: the perichondrium as source of osteoprogenitors for trabecular bone formation

In bones developing by endochondral ossification, the first committed osteoblast lineage cells expressing the early markers *Runx2* and *Osx* appear in the perichondrium surrounding the cartilage anlagen; at least a subset of these cells differentiate to mature, bone-forming osteoblasts that deposit the bone collar, the prelude of the later cortical bone shaft (Maes et al., 2010c). This early osteoblast differentiation program proceeds in tight coordination with the hypertrophic differentiation of chondrocytes in the bone model, through an abundant molecular cross talk between the perichondrium and the underlying cartilage. On the one hand, perichondrial cells produce a myriad of paracrine factors that can influence the proliferation and differentiation of the adjacent chondrocytes, including fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs), and Wnts (Kronenberg, 2007). On their turn, chondrocytes in the bone anlagen and fetal growth plates produce signals, such as Indian hedgehog (*Ihh*), that direct mesenchymal cells in the surrounding perichondrium towards the osteoblast fate by inducing *Runx2* expression (Kronenberg, 2003, Kronenberg, 2007). Conversely, *Runx2* reciprocally also regulates *Ihh* expression and hypertrophic differentiation (Long, 2012). *Ihh*, a member of the conserved family of hedgehog proteins, is produced by the pre-hypertrophic and early hypertrophic chondrocytes of the fetal cartilage, and signals

through the receptor Patched (Ptc) in adjacent chondrocytic and perichondrial domains. Via a negative feedback signaling pathway with parathyroid hormone-related peptide (PTHrP), Ihh regulates the pace of hypertrophic differentiation in the growth plate (Kronenberg, 2003). In addition, localized signaling by Ihh induces osteoblastogenesis and bone collar formation in the perichondrium immediately adjacent to the (pre-)hypertrophic chondrocytes by stimulating the expression of Runx2. Through these combined functions, Ihh orchestrates the spatiotemporal alignment of hypertrophic chondrocyte maturation with perichondrial osteoblast differentiation and bone collar formation (Long, 2012, Kronenberg, 2007, Kronenberg, 2003).

Establishment of the bone collar is associated in time and place with vascular accumulation in the mid-diaphyseal perichondrial region. The cartilaginous tissue that prefigures the future bone itself is intrinsically avascular. As it grows in early development, chondrocytes vigorously proliferate and produce abundant extracellular matrix largely consisting of type II collagen and proteoglycans. At a certain moment, the chondrocytes exit the cell cycle, undergo maturation and finally become hypertrophic; they now typically secrete a matrix rich in type X collagen that partially becomes calcified. At this stage they also produce angiogenic stimuli, such as VEGF (see further), attracting blood vessels to the inner perichondrial tissue layers immediately adjacent to the hypertrophic cartilage (Maes, 2013). These blood vessels will subsequently, around E14.5 in most long bones of the mouse, invade the hypertrophic portion of the cartilage anlagen. As the cartilage matrix becomes resorbed by osteoclasts, osteoblast progenitors populate the region formerly occupied by chondrocytes, differentiate into osteoblasts, and deposit osteoid on cartilage remnants. By E16.5, the middle portion of the cartilage is replaced by the primary ossification center, the highly vascularized region inside the bone shaft occupied by trabecular bone and bone marrow and that will further expand as the bone lengthens. The hematopoietic (stem) cells that are to constitute the bone marrow travel through the blood stream into the bone environment (Christensen et al., 2004), but the origin and entry of the osteogenic stromal cells and osteoblasts giving rise to the trabecular bone compartment inside the long bones was long elusive. Of several hypotheses raised (see below), the perichondrium represented a prime candidate source to provide these cells. In an ex vivo assay using embryonic limb explants grown underneath the renal capsule of adult mice, the perichondrium was shown to deliver cells to the inside of the cultured bones; moreover, if the perichondrium was removed from the explants, they did not develop a primary ossification center and instead remained as entirely cartilaginous rudiments (Colnot

et al., 2004). Lineage tracing studies recently endorsed perichondrial osteoprogenitors as source of osteoblasts for trabecular bone formation in endochondral ossification in vivo (Maes et al., 2010c). Specifically, cells in the perichondrium characterized by expression of the early osteogenic marker *Osx* were found to travel to the inside of the developing bone and give rise to trabecular osteoblasts, osteocytes and stromal cells populating the primary ossification center. The specific tracking of the cells from their site of origin was made possible by the generation of 2 transiently tamoxifen-inducible transgenic mouse lines, carrying Cre-ERT transgenes driven by the *Osx* and *Col1* (3.2 kb) gene promoters that become expressed in osteoprogenitors and mature osteoblasts, respectively. These mice were bred to Rosa26R-LacZ reporter mice to allow their genetic labeling with β -galactosidase (LacZ) expression upon the administration of tamoxifen. A single bolus injection of tamoxifen at E12.5 or E13.5 was successful in marking a number of Cre-ERT-expressing cells in the perichondrium surrounding the avascular cartilaginous bone rudiment prior to its initial vascular invasion, as visualized by staining for the LacZ substrate X-gal. Pulse-chase studies allowed the specific tracing of these cells within the heterogeneous bone context, following their fates during bone development. These studies revealed that between embryonic day E14 and E16, *Osx*/LacZ⁺ osteoprogenitors moved from the perichondrium to the inside of the shaft to initiate the nascent primary ossification center. Some of these cells and their progeny differentiated into mature bone-anchored osteoblasts that became responsible for trabecular bone formation, while another portion remained part of the immature osteogenic stroma in the intertrabecular bone and marrow space. In contrast, similarly labeled perichondrial mature osteoblasts expressing the *Col1*-CreERT transgene (*Col1*/LacZ⁺ cells) mostly stayed in the perichondrial area enveloping the bone shaft and generated the outer cortical bone (Maes et al., 2010c).

These data indicated that stage-selective subsets of osteoblast lineage cells originating from the early fetal perichondrium display differential destinies in developing bones. Osteoprogenitors, in addition to becoming bone collar osteoblasts, are also recruited to the inside of the bone to form the trabecular region, while osteoblasts that matured while residing in the perichondrium predominantly build the cortex (Maes et al., 2010c). These findings do not exclude other potential sources of trabecular osteoblasts in endochondral bone development. For instance, circulating progenitors delivered through the blood stream, chondrocytes, and pericytes, have been and are being considered as cellular sources of the osteoblastic cells that produce trabecular bone (Colnot et al., 2004, Galotto et al., 1994,

Khosla et al., 2010, Roach and Erenpreisa, 1996). To date, however, evidence for and estimates of the respective contributions of these potential osteoblast sources relative to the perichondrial osteoprogenitors is only sparsely available. **Hypertrophic chondrocytes** are generally thought to undergo apoptosis, but the hypothesis that they may exhibit osteoblastic transdifferentiation potential has been proposed decades ago and remains a matter of debate, speculation, and research (Gentili et al., 1993, Galotto et al., 1994, Roach and Erenpreisa, 1996, Shapiro et al., 2005, Hilton et al., 2007). **Pericytes** have been amply proposed as osteoprogenitors and found capable of differentiating into functional osteoblasts (Crisan et al., 2008, Khosla et al., 2010, Sacchetti et al., 2007, Kalajzic et al., 2008). Several primitive mesenchymal cell populations residing in perivascular locations have been recognized in bone, including CXCL12-abundant reticular (CAR) cells, Nestin-expressing cells, and cells marked by expression of *Osx* (in the mouse) and of the cell surface marker CD146 (in adult human bone) or (Mendez-Ferrer et al., 2010, Sugiyama et al., 2006, Sacchetti et al., 2007, Maes et al., 2010c). Such cells may possibly constitute reserve precursors that can be activated later to complete an inherent osteoblast differentiation program.

Particularly intriguing in the osteoblast lineage tracing study discussed above was the finding that the entry of the osteoprogenitors into the primary ossification center coincided with the initial invasion by blood vessels of the intermediate avascular cartilage tissue (Maes et al., 2010c). The labeled *Osx*/*LacZ*⁺ cells co-migrated along with the neovascularization front, with some of them intimately associating with the cartilage-invading blood vessels, being wrapped as pericytes around the endothelium (Figure 3) (Maes et al., 2010c). The molecular underpinnings of this phenomenon are not yet understood. A plausible hypothesis is that the coinciding movement of perichondrial osteoprogenitors and blood vessels into developing bones is triggered by common chemo-attractants, emanating from the degrading late-hypertrophic cartilage matrix and directionally steering the osteo-angiogenic co-invasion. Multiple factors that may be involved in the attraction of osteoblast and/or their precursors to sites in need of new bone formation have been identified. These include chemotactic growth factors that are released, activated or produced at sites of tissue resorption, and in many cases also have angiogenic properties. A second possible hypothesis, and not mutually exclusive, is that interactions between osteoblast lineage cells and endothelial cells determine the joint processes of angiogenesis and osteoprogenitor trafficking into the bone.

The current knowledge on chemo-attractants for osteogenic cells, on the control of osteoblast lineage cell recruitment to sites of active bone formation, and on the regulation of osteo-angiogenic coupling is reviewed here.

CONTROL OF OSTEOLAST RECRUITMENT AND BONE FORMATION DURING DEVELOPMENT AND REMODELING BY CHEMOTACTIC AND OSTEO-ANGIOGENIC COUPLING FACTORS

As mentioned above, during bone development there is a clear spatial-temporal phase to be discriminated where osteogenic cells travel from the outer perichondrium to the developing primary ossification center inside the bone. Cell migration is a dynamic process that requires the coordinated formation and disassembly of focal adhesions and re-arrangements of the actin cytoskeleton. Although the underlying mechanisms are still poorly understood and will not be covered here, there is evidence that the osteoprogenitors are attracted, together with blood vessels, by chemotactic factors released from the mold. But also in the adult skeleton, when bone is being constantly remodeled, osteoblasts or their precursors need to be recruited to the active bone surfaces. This phase in the remodeling cycle is not only imperative for bone health, but may also bear promising potential towards the development of therapeutic strategies for conditions where anabolic stimulation of bone formation is sought, such as in osteoporosis. Intriguingly, a tight correlation between ossification and vascularization of bone remains evident, also in remodeling bones beyond the stages of development and growth. Recent data have started to identify some key factors that may be involved in the regulation of osteogenic cell chemo-attraction and in the orchestrated interplay between osteoblast recruitment and functioning and angiogenic processes.

Growth factors inducing chemotaxis of osteoblast lineage cells in vitro

Recruitment of osteoblast lineage cells to the sites programmed for ossification or in need of new bone formation conceivably relies on precisely controlled temporal and spatial chemotactic navigation signals. There are multiple candidate guidance cues that may be involved in the directional migration of osteoblast and/or their precursors. In vitro, several factors with chemotactic potential towards osteoblasts and their precursors have been identified (see Table 1). These include, but are not restricted to, constituents of the bone

matrix such as fragments of collagen or osteocalcin, complement fragments, inflammatory cytokines and growth factors that are typically released, activated or produced at sites of matrix resorption (also see below). In this section we will focus primarily on the secreted growth factors, which have been studied most intensively. Examples include platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and its homologue placental growth factor (PlGF), and transforming growth factor β (TGF- β) (see Table 1 and references therein). Among their many other effects on mesenchymal stem and progenitor cells and osteoblasts (such as affecting their proliferation, lineage determination and differentiation, survival, and/or activity), they are potent inducers of osteogenic cell migration in vitro. The effect on cell motility induced by these soluble factors is typically assessed using trans-membrane Boyden chamber systems (also referred to as transwell assays, testing chemotactic attraction of cells cultured on a perforated membrane in an upper well towards a substance added to the lower well) or in vitro wound healing assays ('scratch', '2D cell migration', or 'gap-closure' assay). Osteogenic cells from a variety of sources and at different levels of osteoblastic differentiation have been tested for their chemotactic responses to these growth factors, as listed in Table 1.

PDGF is a major mitogenic agent for mesenchymal cells, primary osteoblasts and various osteoblastic cell lines (Abdennagy et al., 1992, Canalis et al., 1992). In addition, PDGF (particularly PDGF-BB) was found to be a powerful chemotactic factor for mesenchymal cells and osteogenic cells in vitro (see Table 1). PDGF exists as various isoforms (AA, AB, BB, CC and DD), which can bind with different affinities to dimerized receptors consisting of two subunits (PDGFR α and PDGFR β) that assemble either in a homotypic or heterotypic manner. Both receptor subunits are expressed on the cell surface of osteoblasts and their precursors. Chemotactic activity has been reported for PDGF-AA, -BB and -AB, whereas PDGF-CC had no effect (for references, see Table 1). PDGF- DD, the newest member of the family, is not highly expressed in bone and its role in herein remains unclear.

The migration capacities of mesenchymal cells appear in vitro to change during osteogenic differentiation: migration is generally highest at early differentiation stages and gradually decreases during later differentiation while adhesiveness increased (Ichida et al., 2011). This is essentially in line with the abovementioned in vivo study using stage-specific osteoblast lineage cell tracing in which specifically osteoblast precursors, and not mature osteoblasts, were found to migrate from the perichondrium to the developing bone center (Maes et al., 2010c). There are, however, exceptions: while cultured undifferentiated osteoblasts are more

sensitive to PDGF-BB, BMP-2 and BMP-4, more mature osteoblasts were found to respond better to TGF- β 1 and bFGF. This may be related to the differently regulated expression of the various receptors during osteoblastogenesis. For instance, it has been reported that expression of PDGFR α and PDGFR β decrease with differentiation of MC3T3-E1 cells (Beck et al., 2001, Fiedler et al., 2004, Sanchez-Fernandez et al., 2008), while several FGF receptors increase (Beck et al., 2001, Haupt et al., 2009).

In contrast to PDGF, whose in vivo role in the skeleton has not been elucidated, VEGF, PlGF, and TGF- β have been thoroughly studied using in vivo models. **VEGF** is well established as an important regulator of skeletal angiogenesis and osteoblast differentiation, as will be described in-depth in the next section. Of the greater VEGF family (consisting of VEGF-A (referred to as VEGF) to VEGF-E and PlGF, a homologue acting via VEGFR1 or Flt-1, one of the main tyrosine kinase VEGF receptors), several members have been shown to exert chemotactic effects on mesenchymal progenitor cells and osteoblastic cells in vitro (for references, see Table 1). Hence, VEGF represents an outstanding candidate to induce concomitant migration of osteoprogenitors and endothelial cells. Such a combined chemotactic effect may perhaps provide partial explanation of the phenomenon of osteogenic-angiogenic coupling (see below).

TGF- β and a number of BMPs (which belong to the larger TGF- β family) are also well documented to induce osteoblast cell migration and differentiation (Table 1). Moreover, as discussed next, TGF- β was elegantly shown to be important for osteoblast recruitment to bone remodeling sites in vivo (Tang et al., 2009).

Osteoblast recruitment during bone remodeling in vivo

Bone is an extremely dynamic tissue; throughout life, it goes through continuous cycles of osteoclastic resorption and osteoblastic formation of new bone matrix. This process of bone remodeling is needed to remove older matrix and cells and stress-induced microcracks, in order to maintain the integrity and biomechanical stability of the skeleton, and to regulate mineral homeostasis of the whole organism. Bone remodeling takes place at multiple sites throughout the skeleton at each given time, in entities called Bone Remodeling Units (BRU). A given BRU typically consists of osteoclasts that initiate the bone remodeling by removing old bone matrix, and osteoblast lineage cells that subsequently become activated to lay down

new bone. Four phases are discriminated in the bone remodeling cycle: the phases of activation, resorption, reversal and formation. In the first phase, hematopoietic osteoclast precursors proliferate, fuse, differentiate and become activated to form active multinucleated mature osteoclasts; this process is regulated by the combined action of macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL), both expressed by osteoblast lineage cells (see below) (Sims and Gooi, 2008, Henriksen et al., 2009). Once osteoclasts are activated, resorption starts by the local secretion of HCl via H^+ -ATPase proton pumps and chloride channels to lower the pH, and by the release of resorbing enzymes to digest the old bone. In the reversal phase, osteoclast activity ceases and osteoblast precursors are recruited to the BRU, where they differentiate to mature, cuboidal osteoblasts that adhere to the bone surface (Sims and Gooi, 2008, Henriksen et al., 2009). During the subsequent formation phase, the differentiated osteoblasts deposit new bone matrix that becomes mineralized. Osteoblasts that become entrapped within the matrix further differentiate to osteocytes; these cells play important roles in mechano-sensing and the regulation of bone remodeling (Bonewald, 2011).

This 4-stage process of bone remodeling needs to be well balanced and tightly controlled to maintain a normal bone mass. Failure of this homeostatic skeletal turnover is one of the most common early manifestations of aging. In pathologies characterized by an increased bone turnover, osteoblastic bone formation cannot compensate for the rapid osteoclastic resorption. A higher rate of bone remodeling with a net bone loss is the most prevalent mechanism underling the development of widespread low bone mass disorders such as osteoporosis. The most typical and frequent cause of osteoporosis is the postmenopausal decline in estrogen levels in elderly women. Estrogen deficiency increases both the number of sites at which remodeling is initiated, and the extent of resorption at a given site. The most routinely used therapies against osteoporosis are anti-catabolic drugs that effectively inhibit osteoclastic bone resorption, but there is a great clinical demand for anabolic treatments that increase bone formation (Baron and Hesse, 2012, Rachner et al., 2011) (see further).

One potential way to increase osteoblast activity on the bone surface and improve bone formation could be by stimulating the recruitment of osteoblast precursors towards the bone surfaces and bone resorptive sites. During normal bone remodeling, the activities of both osteoclasts and osteoblasts are ‘coupled’ such that the resorption and formation of bone are balanced and the net bone mass is maintained. This balance implies the existence of mechanisms tightly coordinating the differentiation of osteoblasts and osteoclasts as well as

their migration to locations where they function. On the one hand, cells of the osteoblasts lineage directly control osteoclastogenesis and bone degradation, by virtue of the expression of the necessary osteoclastogenic signals M-CSF and RANKL in bone marrow stromal cells (BMSCs), osteoblasts, and osteocytes (Sims and Gooi, 2008, Henriksen et al., 2009, Nakashima et al., 2011). Conversely, osteoclasts are thought to reciprocally stimulate osteoprogenitor recruitment and osteoblast differentiation to initiate the anabolic arm of the remodeling process. With regard to the induction of osteogenic cell recruitment, it is assumed that the process of osteoclastic resorption of the bone matrix mediates the local release of a myriad of growth factors that are stored in the extracellular matrix, which can subsequently act as potent stimuli of osteoprogenitor chemotaxis and thereby couple bone resorption to bone formation. Growth factors like TGF- β , BMPs, IGFs, VEGF and PDGF, discussed above for their potency to attract osteoblastic cells and/or progenitors, are known to have strong affinity for extracellular constituents of bone and to be partly sequestered into the bone matrix after their secretion (Sims and Gooi, 2008). The study by Tang et al. (Tang et al., 2009) provided the first in vivo proof of this longstanding hypothesis by indicating that TGF- β , released from bone matrix during osteoclastic bone matrix degeneration, exerts a chemotactic effect on osteoblast precursors to these bone resorptive sites. In particular, the authors showed that medium conditioned by a culture of osteoclasts on bone slices induced migration of BMSCs in vitro, largely mediated via TGF- β 1; TGF- β promoted BMSC migration and lamellopodia formation through SMAD signaling. Mice lacking TGF- β 1 showed disturbed bone remodeling with decreased trabecular bone volume and thickness, increased trabecular separation, and the presence of fewer osteoblasts on the trabecular bone surfaces at 3 months of age. Most notably, injected osteogenic BMSCs that were labeled with GFP were found to home to the trabecular bone surfaces in WT mice, whereas only very few injected BMSCs migrated to bone resorptive sites in TGF- β knockout mice (Tang et al., 2009).

In addition to chemotactic signals released from the matrix upon resorption, osteoclasts can also play a direct role in regulating osteoblast recruitment. For instance, osteoclasts themselves secrete factors with chemotactic properties such as PDGF (Sanchez-Fernandez et al., 2008), and they can signal directly to cells of the osteoblast lineage through cell-bound molecules (Sims and Gooi, 2008, Henriksen et al., 2009). In a recent paper evidence was provided that osteoclast-produced semaphorin 4D (Sema4D, a predominantly transmembrane protein previously known as axon-guidance molecule) contributes to balanced remodeling in adult bone by binding to its receptor Plexin-B1, expressed on osteoblasts (Negishi-Koga et al., 2011). This interaction in fact appears to repel osteoblasts from osteoclasts at bone

resorptive sites, thus suppressing bone formation during the bone resorption phase. Mice lacking Sema4D or Plexin-B1 or both showed increased bone volume, trabecular thickness and bone strength, resulting from increased osteoblastic bone formation. Although the fine details of the underlying mechanism remain to be clarified, activation of the small GTPase RhoA and its downstream kinase ROCK seem to be implicated in the Sema4D-induced inhibition of osteoblast differentiation and stimulation of cell motility (Negishi-Koga et al., 2011).

New insights in the mechanisms underlying the induction of the reversal phase, during which osteoblasts are recruited to the BRU, may eventually lead to the development of novel therapeutic targets to treat osteoporosis.

Bone formation is coupled to vascularization: regulation by hypoxia signaling and VEGF

The recruitment of osteoprogenitors into new bone centers forming via endochondral ossification, both in developing and healing bones, coincides with neovascularization of the cartilage intermediate (Maes et al., 2010c). Also intramembranous bone formation occurs is tightly associated with angiogenesis and vascularization of the forming bone. Moreover, mature bone is highly vascularized, and the lifelong process of bone remodeling in the BRUs occurs in close proximity of the blood vessels of the bone and bone marrow environment. Reduced blood flow has been linked to old age and low bone mass disorders such as osteoporosis, and to impaired fracture healing (see below) (Andersen et al., 2009, Lafage-Proust et al., 2010, Maes, 2013, Burkhardt et al., 1987).

This close spatial and temporal association of bone formation with vascularization, which is well recognized in virtually all settings of ossification, has been termed ‘angiogenic-osteogenic coupling’ (Schipani et al., 2009, Wan et al., 2010). The reasons that the vascular system is crucial for bone formation during skeletal development, maintenance and repair obviously include its intrinsic function to supply the necessary oxygen, nutrients and growth factors/hormones to the bone cells. The blood vessels also deliver hematopoietic precursors of osteoclasts to sites of cartilage and bone resorption and remove end products of the extracellular matrix degradation. In addition, the vasculature likely serves to bring in progenitors of osteoblasts for subsequent bone depositing. For instance, the subendothelial wall of skeletal blood vessels contains pericytic cells consistent with osteoprogenitors in

terms of molecular profile and osteoblastic differentiation potential; this could well offer part of the cellular explanation for the coupling paradigm between angiogenesis and osteogenesis (Maes et al., 2010c, Sacchetti et al., 2007). Molecularly, the precise mechanisms of angiogenesis and its coupling with osteogenesis during bone development, fracture healing and bone remodeling are not completely understood, but studies using a variety of genetic mouse models identified VEGF and hypoxia signaling components as key players of angiogenic-osteogenic coupling (Table 2) (Schipani et al., 2009, Maes et al., 2012b, Riddle et al., 2009).

Regulation of bone vascularization by VEGF: lessons from genetically modified mouse models

Vascularization is an absolute requirement for bone development, growth, repair and homeostasis. This is particularly evident during endochondral ossification processes: the cartilage intermediate remains as an avascular mesenchymal tissue until the chondrocytes reach a fully differentiated state of hypertrophy. Terminal hypertrophic cartilage undergoes angiogenic invasion, a process that is associated with the decay of the cartilage matrix and its replacement by bone. During development, three consecutive vascularization stages can be delineated that altogether cover the replacement of the avascular cartilage template by highly vascularized bone and marrow tissue (see Figures 1 and 3) (Maes, 2013). First, the initial vascular invasion of the cartilage anlagen in the early embryo involves endothelial cells invading from the perichondrial tissues and organizing into immature blood vessels in the primary center of ossification. Second, capillary invasion at the metaphyseal border of the growth cartilage mediates rapid bone lengthening. Third, vascularization of the cartilage ends initiates the formation of secondary ossification centers. Each of these vascularization events is closely followed by ossification in the respective area (the primary ossification center, trabecular bone formation at the metaphyseal chondro-osseous junction, and the secondary ossification center, respectively). Hence, the vascularization of the expanding bone centers is a crucial aspect of bone development and growth. Early in vivo experiments exposed the importance of the vasculature to growing bones by inducing physical blockage of the bone's blood supply, resulting in reduced longitudinal growth (Trueta and Morgan, 1960). Molecularly, VEGF (VEGF-A) is one of the most powerful and critical mediators of angiogenesis. The deletion of even a single copy of the *Vegf* gene results in early embryonic lethality owing to defective vascular development. Five VEGF isoforms have been identified

in humans, while there are three major isoforms in the mouse (VEGF120, VEGF164, and VEGF188). VEGF binds to and activates two tyrosine kinase receptors, VEGFR1 (Flt-1) and VEGFR2 (KDR/Flk-1), which regulate both physiological and pathological angiogenesis. In addition, neuropilins (NRP1 and NRP2) can act as co-receptors for specific VEGF isoforms (Yla-Herttuala et al., 2007).

In endochondral bones, VEGF is expressed abundantly by late hypertrophic chondrocytes, where it is critical for blood vessel invasion and replacement of cartilage by bone. This was first shown by the inhibition of VEGF action in juvenile (24-day-old) mice via administration of a soluble VEGF receptor chimeric protein (mFlt (1-3)-IgG or sFlt-1): vascular invasion of the growth plate became impaired and concomitantly, trabecular bone formation and bone growth were reduced. Also, the hypertrophic cartilage zone became enlarged, likely due to reduced osteoclast-mediated resorption (Gerber et al., 1999). Further studies performed over the last decade using genetically modified mice have univocally established that VEGF is an essential physiological mediator of all 3 key vascularization stages of endochondral bone development. Given the fact that a full knockout of VEGF led to early lethality even in the heterozygous state, several alternative mutagenesis approaches have been and are being exploited to investigate the role of VEGF in the skeleton. These models include the Cre/LoxP-mediated conditional inactivation of the VEGF gene (*Vegfa*) in specific skeletal cell subsets, genetically engineered mice expressing only one of the 3 major VEGF isoforms, and transgenic over-expression strategies making use of temporal and/or tissue-specific mutagenesis. Table 2 gives an overview of studies employing genetic mouse models of perturbed expression of VEGF family members in the skeleton and their phenotypic outcome (see Table 2 and references therein).

Altogether, these models have exposed multiple essential roles of VEGF and its isoforms in endochondral ossification, not only as key inducer of vascularization but also as a direct modulator of bone development by affecting the various cell types involved. Mesenchymal stem and progenitor cells, perichondrial cells, osteoblasts and osteoclasts all express both VEGF and VEGF receptors and respond to VEGF signaling by enhanced recruitment, differentiation, activity and/or survival (reviewed in (Maes, 2013, Dai and Rabie, 2007)). These pleiotropic actions of VEGF on the various cells in the bone environment contribute to the tight coordination of vascularization, ossification, and matrix resorption in endochondral ossification. The current model (see Figure 3) is that VEGF is produced at low levels by immature chondrocytes, where it stimulates chondrocyte survival at least in part by inducing perichondrial vascularization. VEGF is also produced at high levels by hypertrophic

chondrocytes and is partly sequestered in the cartilage matrix upon its secretion. Trapped VEGF (hence, particularly the isoforms that have high matrix-binding affinity, such as VEGF₁₆₄ and VEGF₁₈₈) can be released from the matrix by proteases like matrix metalloproteinase (MMP)-9, secreted by osteoclasts/chondroclasts during cartilage resorption. VEGF can then bind to its receptors on endothelial cells and stimulate the directional growth of angiogenic blood vessels to invade the hypertrophic cartilage, which may be indirectly associated with an increased potential delivery of osteoblast and osteoclast progenitors. The osteoblasts or their precursors in this region are also affected by the VEGF signaling in their recruitment, proliferation, differentiation and/or function. At the same time, VEGF directly works as a chemo-attractant stimulating osteoclast invasion of cartilage and enhancing osteoclast differentiation, survival and resorptive activity, thereby creating a positive feedback system through the release of more matrix-bound VEGF from the cartilage that is being resorbed (reviewed in (Maes, 2013)).

The role of VEGF as a prime mediator of angiogenic-osteogenic coupling was revealed in most or all of the mutant mouse models listed in Table 2. Reduced VEGF signaling was shown to reduce both vascular density and bone formation in loss-of-function mice (Maes et al., 2002, Gerber et al., 1999). Conversely, temporal VEGF over-expression in the skeleton induced combined hyper-vascularization and increased bone formation during development and in adult mice (Maes et al., 2010b). Besides the indirect, vascularization-mediated influences on bone formation, direct VEGF-effects on osteogenic cells also contribute to the coupling. Although the relative importance of these aspects is extremely difficult to delineate *in vivo*, several *in vitro* studies provided evidence for cell-autonomous roles of VEGF signaling in stimulating chemotactic migration of osteogenic cells (see Table 1) and affecting osteoblastic proliferation and differentiation (Hah et al., 2011, Mayer et al., 2005, Grellier et al., 2009). In addition to autocrine and paracrine signaling of extracellular VEGF, intracellular VEGF was recently implicated to act via intracrine ways in osteogenic cells (Liu et al., 2012). Conditional deletion of VEGF in osteoprogenitors induced an osteoporosis-like phenotype characterized by reduced bone mass and increased bone marrow fat. VEGF knockdown in cultured BMSCs further revealed that osteogenic differentiation was reduced in the absence of intracellular-acting VEGF, to the benefit of adipogenesis. Thus, intracrine VEGF plays a role in the regulation of the balance between osteoblast and adipocyte differentiation (Liu et al., 2012).

The actions of VEGF, also in the skeleton, are highly dose-dependent, and its physiological levels must be under very strict control mechanisms. Several hormones (including PTH, GH, $1,25(\text{OH})_2\text{D}_3$), locally produced growth factors (e.g. FGFs, TGF- β , BMPs, IGFs, PDGF), and skeletal transcription factors (including Runx2 and Osx) have been implicated in the regulation of VEGF expression (Tang et al., 2012, Maes, 2013, Dai and Rabie, 2007). One of the best-characterized regulatory mechanisms upstream of VEGF expression, in general and in skeletal tissues, is hypoxia signaling via the hypoxia-inducible factor (HIF) pathway.

HIF pathway involvement in osteo-angiogenic coupling

Cellular oxygen-sensing mechanisms play major roles in pathological settings such as cancer and ischemia, and in normal development and homeostasis. HIFs (HIF-1 and HIF-2) are ubiquitously expressed transcription factors that modulate gene expression to mediate cellular responses and adaptation in hypoxic environments; typical targets include genes involved in glucose utilization and cell metabolism (stimulating anaerobic glycolysis), angiogenesis (for instance by inducing VEGF expression, a direct target of HIF-1), and erythropoiesis (through increased erythropoietin (EPO) production) (Semenza, 2012). HIFs are heterodimers consisting of an α -subunit, which is regulated by oxygen, and a β subunit that is constitutively expressed in an oxygen-independent manner. When sufficient oxygen is available, HIF-1 α (as well as HIF-2 α) becomes hydroxylated on specific amino-acid residues within its oxygen-dependent degradation domain (ODD) by prolyl hydroxylases. This hydroxylation renders HIF-1 α to be recognized by the Von Hippel-Lindau protein (pVHL), an E3 ubiquitin ligase, and targeted for degradation in the proteasome. VHL thus functions as an upstream negative regulator of HIF, by affecting protein stability. In hypoxic conditions this hydroxylation of HIF-1 α does not occur, and HIF-1 α protein can accumulate, translocate to the nucleus, dimerize with HIF-1 β , and bind to hypoxia response elements (HRE) within the promoters of its hypoxia-responsive target genes (Semenza, 2012).

Hypoxia too is one of the drivers of the tight coupling between angiogenesis and osteogenesis in skeletal tissues, at least in part through induction of VEGF. Hypoxia is a strong trigger of VEGF expression in chondrocytes, osteoblasts, and possibly osteoclasts, through mechanisms that involve HIF both in vitro and in vivo (see (Schipani et al., 2009, Riddle et al., 2009, Maes et al., 2012b)). The role of HIF pathway components in the skeleton has been studied using a growing collection of mutant mouse models, primarily Cre-loxP-mediated conditional knockouts (listed in Table 2). Their functioning as key regulators of osteo-angiogenic

coupling in adult bone was first revealed in a study by Wang et al (Wang et al., 2007), who generated VHL and HIF-1 α conditional knockout mice using a Cre recombinase expressed under the control of the osteocalcin gene promoter, expressed in differentiated osteoblasts. Upon disruption of the Vhl gene, HIFs were stabilized and the level of VEGF expression increased. Micro-CT analysis showed a massive increase in bone volume and osteoblast number and a decrease in trabecular separation. In vitro, osteoblast differentiation was not altered upon VHL inactivation and no changes in proliferation rate and apoptosis were detected, suggesting that osteoblastic HIFs may exert their effect in a cell-nonautonomous manner. The increase in bone mass was associated with a proportional increase in vascular density, correlating with upregulation of VEGF. The opposite phenotype was observed when HIF-1 α was inactivated in mature osteoblasts. A double knockout of VHL and HIF-1 α still caused vascular effects similar to those in the VHL knockout mice as well as a milder but evident increase in trabecular and cortical bone mass, which was attributed to HIF-2 α taking over the function of HIF-1 α in its absence, to enhance VEGF production and drive angiogenesis (Wang et al., 2007). As such, this study allocated a central role to HIF-1 α in coupling angiogenesis to osteogenesis during endochondral ossification. In a more recent study, the promoter of the earlier osteogenic marker Osx was used to direct a conditional knockout of VHL, leading to the discovery of a role for HIF signaling (predominantly through HIF-2) in osteoprogenitors in controlling hematopoiesis, particularly red blood cell production, in an EPO-dependent manner. Indeed, inactivation of VHL in osteoprogenitors led to increased EPO expression in bone and selective expansion of the erythroid lineage, while inactivation of HIF-2 α , but not HIF-1 α , reduced EPO expression in bone (Rankin et al., 2012). Thus, osteoblast lineage cells emerged as important regulators of the coupling by sensing oxygen tension and directing adjustments in vascularization and oxygen supply to meet their demands for optimal ossification (Schipani et al., 2009, Maes et al., 2012b, Maes, 2013, Riddle et al., 2009).

The hypoxia-inducible cascade, particularly HIF-1 α and VEGF, also plays critical roles in the early development of the long bones (Figure 3). Both have been implicated in the initial osteo-angiogenic co-invasion of developing bones in vivo and in osteogenic cell migration in vitro. Immature chondrocytes in the early endochondral cartilaginous condensations are resistant to vascular invasion due to the production of angiogenic inhibitors, such as chondromodulin-I, troponin-I and thrombospondins (see (Maes, 2013)). As a result, the chondrocytes of the long bone anlagen and fetal growth plates experience hypoxia (Schipani

et al., 2001, Maes et al., 2004). The chondrocytes are capable to survive and proliferate and differentiate in this hypoxic environment, at least in part via HIF-regulated adaptation mechanisms to oxygen deprivation (Maes et al., 2012a, Schipani et al., 2001). Inactivation of HIF in chondrocytes causes cell death due to the inability of the cells to switch to vital oxygen-sparing metabolic pathways (HIF-dependent activation of anaerobic glycolysis), and by the lack of sufficient blood vessel accumulation in the perichondrium immediately surrounding the cartilage (VEGF-dependent angiogenesis). In mice over-expressing VEGF (Col2-Cre mediated VEGF164 conditional transgenic) osteo-angiogenic invasion of developing bone centers occurred prematurely and excessively, with aberrant bone deposition in this vascularized area leading to misshapen limbs (Maes et al., 2010b). Conversely, the invasion process and primary ossification center formation was delayed in mice lacking HIF-1 α or VEGF in cartilage, which could be rescued by forced VEGF164 over-expression in the combined mutant (in contrast to the cell death of HIF-deficient chondrocytes that could only be partially rescued) (Maes et al., 2012a). These findings strongly suggest that hypoxia-induced, HIF-mediated VEGF expression in cartilage is required for the concomitant recruitment of angiogenic blood vessels and osteoprogenitors into developing endochondral bone centers (Figure 3). As mentioned previously, VEGF is chemo-attractive for MSCs and osteoblastic cells in vitro. Besides VEGF also several other growth factors, including PDGF and TGF- β , have both angiogenic properties and chemotactic effects on osteogenic cells, which could provide partial explanation of osteogenic-angiogenic coupling by a mutual attraction of endothelial cells and osteoprogenitors. These signals are also produced by osteoblast lineage cells themselves, implicating a complex crosstalk between the two cell types in the interplay between osteogenic cell recruitment to new sites of bone formation and vascular invasion and expansion. Moreover, hypoxia signaling by downstream mechanisms other than VEGF has also been shown to affect the migration properties of MSCs. Conditioned medium of MSCs cultured in hypoxic conditions stimulated MSC migration more than medium of normoxic cultures (Annabi et al., 2003). The medium of hypoxic osteocytes also significantly increased MSC migration, which was attributed to the chemotactic effect of hypoxia-induced osteopontin, acting possibly via one of its receptors, β 1 integrin (Raheja et al., 2008). Integrins are important in cell-matrix adhesion; in hypoxic conditions their expression patterns are altered and associated with increased migration of hMSCs (Saller et al., 2012). Although many studies show a positive correlation between hypoxia and migration (Raheja et al., 2008, Saller et al., 2012, Vertelov et al., 2013), some controversy exists. Some studies demonstrate an increased activation of RhoA GTPases in

hypoxic conditions with enhanced targeted migration of MSCs (Vertelov et al., 2013) while others negatively correlate hypoxia with MSC migration through modulation of RhoA activity (Raheja et al., 2011). Further in vitro and in vivo studies will be required to decipher the mechanisms of osteogenic cell migration and recruitment that are regulated by the hypoxia signaling pathway and VEGF.

In conclusion, several molecules have been related to aspects of osteogenic cell recruitment to sites of bone formation and the close coupling of ossification with angiogenic processes. The interactions that tune these orchestrated processes are however still incompletely understood. The interplay between osteogenic cells and endothelial cells is complex and reciprocal, in that the cells of bone on the one hand depend on the signals, oxygen, and cellular precursor delivery via the blood stream, while on the other hand they influence the vascular arrangement and function, for instance through oxygen sensing and angiogenic signaling. The characterization of the molecular machinery controlling these intricate processes relied heavily on the use of mutant mice over the last decade, and its future refinement will depend on the further optimal use of genetic tools to target genes in specific cells and developmental time points. Elucidating further the functional relevance and molecular regulation of osteogenic cell migration and angiogenic-osteogenic coupling mechanisms represents a major challenge, with high potential to offer novel therapeutic avenues for bone diseases and bone regeneration strategies.

RECAPITULATION OF EMBRYONIC MECHANISMS DURING ENDOCHONDRAL BONE REPAIR: OSTEOLAST RECRUITMENT COUPLED TO NEOVASCULARIZATION

Unlike soft tissues, which repair predominantly through the production of fibrous scar tissue at the site of the injury, the skeleton possesses an astounding capacity to regenerate upon damage. Bone defects heal by forming new bone that is indistinguishable from adjacent, uninjured bone tissue. It has been appreciated for a long time that fracture repair in the adult is a multi-step process that bears close resemblance to fetal skeletal tissue development, with both intramembranous and/or endochondral bone formation processes occurring depending on the type of fracture (Schindeler et al., 2008, Beamer et al., 2009). In recent years this close resemblance has been supported by genetic and molecular studies showing that similar

pathways are at work in both settings (Gerstenfeld et al., 2003, Ferguson et al., 1998), although additionally, some signaling molecules that are dispensable for development become important during fracture repair (Maes et al., 2006, Tsuji et al., 2006).

Following bone injury, a hematoma is formed, platelets are activated and degranulate, and acute inflammatory cells and macrophages are recruited to the fracture site. These early inflammatory responses are associated with the release of a plethora of growth factors, including PDGF, VEGF, TGF- β , BMPs, FGFs, and IGFs and cytokines such as TNF- α and interleukins (Ferguson et al., 1998, Gerstenfeld et al., 2003, Schindeler et al., 2008). These signals have important roles in the initiation of the repair process, most likely including in the recruitment of cells with osteogenic potential to repair the defect as several of them have established chemotactic potential *in vitro* (see Table 1). While the blood clot is being stabilized, new blood vessels begin to form by endothelial cells from the periosteal vessels and surrounding muscles. Mesenchymal progenitor cells are recruited to repopulate the site of injury and differentiate into chondrocytes (particularly in the internal callus, i.e. domains distant from ingrowing capillaries) or into osteoblasts (predominantly in subperiosteal areas creating a hard external callus bridging the fracture gap via intramembranous ossification). The (fibro-)cartilage in the soft callus is gradually replaced with immature woven bone, that in the final phase of the healing process is remodeled by the actions of osteoclasts and osteoblasts, ultimately restoring the pre-injury shape, strength and function of the bone (Figure 4).

Like in developing bones, recruitment of osteogenic progenitor cells to the site of new bone formation in the adult setting of endochondral bone repair occurs in close association with neovascularization of the cartilaginous callus tissue (Maes et al., 2010c). In further resemblance of the embryonic perichondrial progenitor cell source, adult *Osx*-expressing osteoprogenitors were markedly abundant in the peripheral portion of the callus initially, apparently originating from the periosteum and migrating inwards (Maes et al., 2010c). Lineage analyses have shown before that the periosteum is a crucial source of osteoblasts and chondrocytes for fracture repair, along with the bone marrow neighboring the callus delivering MSCs to the site (Colnot, 2009). Circulating stem cells or bone marrow cells recruited from distant bones can also be attracted to sites of bone repair or regeneration, for instance by the CXCR4/stromal cell-derived factor-1 pathway, but their contribution to normal healing processes is still to be clarified (Pignolo and Kassem, 2011, Otsuru et al., 2008, Taguchi et al., 2005). For therapy, bone marrow derived MSCs are among the most

patent sources of cells for human therapy, although periosteal cells have gained a lot of interest as promising population for applications in tissue engineering and bone regeneration settings (Bueno and Glowacki, 2009, Khosla et al., 2010).

As the co-invasion of healing bone centers by osteoblast lineage cells and angiogenic blood vessels was studied by confocal microscopy, again their close juxtaposition and the pericytic behavior of a subset of the translocating osteoprogenitors were observed (Maes et al., 2010c) (Figure 4). Neovascularization of cartilage precedes new bone formation in defects that heal via endochondral ossification and is an essential aspect of successful bone repair; failure to restore the vascular network delays or impairs bone healing, as prevalently associated with advanced age and disorders such as diabetes (Schindeler et al., 2008, Beamer et al., 2009, Lienau et al., 2009). Among many crucial bone-anabolic stimuli brought to the callus by the blood vessels, it will thus be of great interest to elucidate how the vasculature provides a conduit for entry of osteoprogenitor cells in the callus and whether this occurs through similar mechanisms as those operating in fetal life (Maes et al., 2010c, Khosla et al., 2010, Kumar and Ponnazhagan, 2012). Most or all of the chemotactic and osteo-angiogenic signals discussed previously in this review and listed in Table 1, are present in the fracture callus. Moreover, the vascular damage following injury of bone disrupts the blood flow, and leads the fracture site to become extremely hypoxic, further boosting VEGF expression. VEGF is strongly increased locally in the fracture hematoma and is also systemically elevated in injured patients (Beamer et al., 2009). Inhibition of endogenous VEGF activity, by sequestration or VEGF receptor blockade, inhibited bone repair and resulted in non-unions in experimental loss-of-function models (Jacobsen et al., 2008, Street et al., 2002). As in development, VEGF secretion in the bone environment attracts blood vessels and stimulates endothelial cells to form new blood vessels, which is likely indirectly associated with an increased potential delivery of mesenchymal stem or progenitor cells with osteogenic potential, from extraluminal sources and/or fracture-mobilized circulating cells (Khosla et al., 2010, Kumar and Ponnazhagan, 2012, Pignolo and Kassem, 2011). VEGF signaling can also directly stimulate the recruitment and differentiation of osteoblasts, bone formation can be further enhanced by endothelial production of osteogenic differentiation factors, such as (VEGF-induced) BMP-2 (Bouletreau et al., 2002). The presence or absence of a functional vascular bed may, along with and probably in part determined by the mechanical stability of the fracture, also determine mesenchymal stem cell fate decisions. Instable, hypoxic fractures appear to favor the formation of avascular cartilage and healing via an endochondral

ossification process. In contrast, when stabilization of the bone segments is sufficient, healing occurs primarily through intramembranous ossification, feasibly supported by the vascular delivery of oxygen, nutrients and trophic factors that permit direct differentiation of mesenchymal cells into osteoblasts (Schindeler et al., 2008, Colnot et al., 2003).

As the evidence supported a crucial role for VEGF in bone healing, it started to be vigorously tested as a potential bone regeneration therapy in a myriad of preclinical models (Keramaris et al., 2008). Administration of VEGF was indeed found to result in increased vascularity and accelerated bone healing (Jacobsen et al., 2008, Street et al., 2002, Beamer et al., 2009, Keramaris et al., 2008), particularly when supplied in combination with BMPs (Kumar et al., 2010, Peng et al., 2002). Notwithstanding the successes, the high hopes for VEGF-based therapies appear to have dampened somewhat by some serious limitations. For example, increased levels of VEGF in the skeleton may bear a substantial risk of local side effects in the bone and bone marrow environment, including disturbances in hematopoiesis (Maes et al., 2010a). Indeed, temporal VEGF over-expression in the long bones of adult mice (via an inducible and tissue-specific ‘gain-of-function’ approach of transgenic VEGF164) quickly induced combined hyper-vascularization and increased bone formation, which however started to obliterate the marrow cavity and cumulated in a severe pathology of osteosclerosis, bone marrow fibrosis and hematological anomalies after only two weeks (Maes et al., 2010a). Moreover, preclinical pro-angiogenic VEGF studies in cardiovascular medicine suggest risks for adverse vascular effects upon systemic leakage (Yla-Herttuala et al., 2007). Perhaps the actions of VEGF itself may be too strong to allow fine-tuned control of its use in the delicate bone micro-environment; other, milder and safer therapy options are therefore being considered for osteo-angiogenic stimulation in fractures (see (Maes et al., 2012b). For instance, data suggest that the VEGF homolog PlGF could be a valuable alternative (Maes et al., 2006), as would modulation of VEGF signaling by affecting upstream regulators such as HIFs and the prolyl hydroxylases that normally target HIF-1 α for degradation (Shen et al., 2009, Wan et al., 2008).

Further in-depth analyses of the mechanisms and regulation of osteogenic cell recruitment and angiogenesis during fracture healing will undoubtedly help providing new therapies for patients with failing repair and new angles in tissue engineering applications.

PERSPECTIVES

The recruitment of osteoblasts to the site of bone formation is essential for skeletal development, remodeling and healing. In developing bones, osteoprogenitor cells need to invade the cartilage mold to form an ossification center. Similarly, osteogenic cells need to populate the callus that is formed following an injury and, during bone maintenance, osteoblasts must relocate to the sites of active bone remodeling. All these processes rely on controlled temporal and spatial navigation cues for the cells to find their way to the sites in need of new bone formation. As outlined in this review, cellular mechanisms and molecular pathways related to processes of in vitro chemo-attraction and in vivo recruitment of osteogenic progenitor cells to developing, remodeling, and healing bones are increasingly being documented. The further in-depth characterization of these processes will open the door to explore how this process can be modulated for anabolic treatment of metabolic bone disorders such as osteoporosis, and in fracture repair and tissue engineering.

Osteoporosis is a very widespread disease that is typified by a decrease in bone mass, severe trabecular and cortical porosity and increased bone fragility. Osteoporosis is associated with a large incidence of debilitating fractures, particularly of the vertebrae and long bones, associated with high morbidity and mortality by virtue of complications. Already millions of people worldwide suffer from osteoporosis and this condition becomes increasingly prevalent with the ageing of the general population. The most frequent cause is the postmenopausal decline in estrogen levels in women; but also aging men can develop osteoporosis. Current estimates predict that one in three women and one in five men older than fifty will sustain an osteoporotic fracture, associated with pain, disability, and even death (Ström, 2011).

The underlying basis of osteoporosis is imbalanced bone remodeling, with osteoclastic bone resorption exceeding osteoblastic bone formation. The most routinely used therapies against osteoporosis are anti-catabolic drugs such as bisphosphonates or the RANKL inhibitor denosumab, which inhibit the resorptive activity or the formation of osteoclasts ^{2,4}. While these therapies are very successful in inhibiting excessive bone remodeling, they also imply a risk for slowing down (micro-) fracture repair. Moreover, they are unable to reverse the disease as the lost bone does not become replenished. A great need for anabolic treatments that increase bone formation therefore currently prevails. Few anabolic therapies exist, such as teriparatide, a parathyroid hormone (PTH)-based therapy, and, although still in preclinical studies, an inhibitor of the Wnt signaling antagonist sclerostin (Baron and Hesse, 2012, Rachner et al., 2011). But the clinical demand for new and better options is high; therefore, a great deal of interest and ongoing research worldwide is aimed at discovering safe and

effective therapies focusing on anabolic pathways. Profound understanding of the cellular and molecular mechanisms mediating bone formation during bone development and remodeling, including the regulatory control of osteogenic cell recruitment to these sites, can help towards this goal.

New osteo-anabolic therapies are not only of interest in fracture prevention, but also to help improve bone healing and (tissue-engineered) bone regeneration applications. Indeed, each year millions of patients suffer a bone fracture (about 6.2 million in the USA alone), comprising a major worldwide health problem associated with a huge burden on healthcare (Franceschi, 2005, Felsenberg et al., 2002). This number is only expected to rise in the future, as a consequence of the increasing ageing of the population and the large prevalence of low bone mass disorders such as osteoporosis. Bone intrinsically possesses an astounding capacity to regenerate upon damage, and generally, bone repair is a rapid and efficient process. However, in about 10% of the patients fracture healing fails, leading to delayed- or non-unions. The causes of impaired healing often are unknown, but excessive damage to the periosteum (a crucial site of osteoprogenitor delivery to the healing tissue), advanced age, systemic disorders, or infection of the injury site are considered important risk factors. These problematic healing conditions pose a significant problem in orthopedics, because standard treatment options such as surgical intervention stabilizing the bone segments by fixation devices and/or reconstructive procedures using autologous bone grafts are often not feasible, for instance when the bone is too brittle and fixation devices and bone grafting procedures will be ineffective. Treatment of patients with failing fracture repair is consequently very challenging. The development of effective therapies for bone regeneration thus poses one of the most clinically and economically important long-term goals of research in the bone field (Franceschi, 2005).

Current research exploring tissue-engineering-based strategies for bone regeneration therapy attempt to mimic events occurring during normal bone development and repair, where multiple factors interact in a defined temporal and spatial sequence (Weiss et al., 2012, Franceschi, 2005). More than 100 clinical trials on bone regeneration are presently in the pipeline (www.clinicaltrials.gov), implementing new findings from basic science to be translated into experimental, pre-clinical applicability tests (Gomez-Barrena et al., 2011). Tissue engineering strategies generally focus on the use of porous, biodegradable, 3-dimensional carriers or ‘scaffolds’ providing structural support and/or osteo-inductive cues. These scaffolds are combined with delivery of growth factors, gene therapy protocols, and the

use of stem cell based therapies to form viable bone grafts and achieve the best possible biologic outcome. Stem cell technology applications usually include human MSCs isolated from the periosteum or bone marrow. Yet, one of the major challenges in tissue engineering is to efficiently and safely expand and manipulate the progenitor cells in vitro and seed them onto the scaffolds before re-administration to the patient. Hence, the success of using tissue-engineering constructs might be significantly increased if strategies would be implemented that stimulate the recruitment of (host-derived) osteoprogenitors to the site of the bone defect. Understanding the mechanisms of osteoprogenitor recruitment into developing and healing bones of animal models could be a vital step towards the development of new, biologically based therapies for the treatment of currently unmanageable skeletal injuries.

More in vitro and in vivo experiments will be needed to expand this knowledge. Further studies of osteogenic cell migration and recruitment to active sites of bone formation will be greatly helped by sophisticated new and improved methods for cell visualization and tracing, such as in vivo live imaging, fluorescent and confocal microscopy methods, that can be combined with cell-specific and/or transiently inducible mutagenesis and reporter activation in transgenic mouse models.

ACKNOWLEDGEMENTS

We gratefully acknowledge funding received from the European Research Council (ERC, Starting Grant 282131, to CM) under the European Union's Seventh Framework Programme (FP7/2007-2013); MVH is a postdoctoral fellow of the Fund for Scientific Research of Flanders (FWO). We have no financial conflicts of interest to disclose.

REFERENCES

- Abdennagy B, Hott M, Marie PJ. 1992. Effects of platelet-derived growth factor on human and mouse osteoblastic cells isolated from the trabecular bone surface. *Cell Biol Int Rep* 16: 235-247.
- Akiyama H, Kim JE, Nakashima K, et al. 2005. Osteo-chondroprogenitor cells are derived from Sox9 expressing precursors. *Proc Natl Acad Sci USA* 102: 14665-14670.
- Amarilio R, Viukov S, Sharir A, et al. 2007. Hif1alpha regulation of Sox9 is necessary to maintain differentiation of hypoxic prechondrogenic cells during early chondrogenesis. *Development* 134: 3917-3928.
- Andersen TL, Sondergaard TE, Skorzynska KE, et al. 2009. A physical mechanism for coupling bone resorption and formation in adult human bone. *Am J Pathol* 174: 239-47.
- Annabi B, Lee YT, Turcotte S, et al. 2003. Hypoxia promotes murine bone-marrow-derived stromal cell migration and tube formation. *Stem Cells* 21: 337-347.
- Araldi E, Khatri R, Giaccia AJ, et al. 2011. Lack of HIF-2alpha in limb bud mesenchyme causes a modest and transient delay of endochondral bone development. *Nat Med* 17: 25-26.
- Augello A, Kurth TB, De Bari C. 2010. Mesenchymal stem cells: a perspective from in vitro cultures to in vivo migration and niches. *Eur Cell Mater* 20: 121-33.
- Baron R, Hesse E. 2012. Update on Bone Anabolics in Osteoporosis Treatment: Rationale, Current Status, and Perspectives. *The Journal of clinical endocrinology and metabolism*.
- Beamer B, Hettrich C, Lane J. 2010. Vascular Endothelial Growth Factor: An Essential Component of Angiogenesis and Fracture Healing. *HSS J* 6: 85-94.
- Beck GR, Jr., Zerler B, Moran E. 2001. Gene array analysis of osteoblast differentiation. *Cell Growth Differ* 12: 61-83.
- Bentovim L, Amarilio R, Zelzer E. 2012. HIF1alpha is a central regulator of collagen hydroxylation and secretion under hypoxia during bone development. *Development* 139: 4473-4483.
- Bonewald LF. 2011. The amazing osteocyte. *J Bone Miner Res* 26: 229-38.
- Bouletreau PJ, Warren SM, Spector JA, et al. 2002. Hypoxia and VEGF up-regulate BMP-2 mRNA and protein expression in microvascular endothelial cells: implications for fracture healing. *Plast Reconstr Surg* 109: 2384-97.
- Bueno EM, Glowacki J. 2009. Cell-free and cell-based approaches for bone regeneration. *Nat Rev Rheumatol* 5: 685-97.

- Burkhardt R, Kettner G, Bohm W, et al. 1987. Changes in trabecular bone, hematopoiesis and bone marrow vessels in aplastic anemia, primary osteoporosis, and old age: a comparative histomorphometric study. *Bone* 8: 157-64.
- Canalis E, Varghese S, McCarthy TL, Centrella M. 1992. Role of platelet derived growth factor in bone cell function. *Growth Regul* 2: 151-5.
- Case N, Rubin J. 2010. Beta-catenin--a supporting role in the skeleton. *J Cell Biochem* 110: 545-53.
- Chen J, Long F. 2013. beta-catenin promotes bone formation and suppresses bone resorption in postnatal growing mice. *J Bone Miner Res* 28: 1160-9.
- Christensen JL, Wright DE, Wagers AJ, Weissman IL. 2004. Circulation and chemotaxis of fetal hematopoietic stem cells. *PLoS Biol* 2: E75.
- Colciago A, Celotti F, Casati L, et al. 2009. In Vitro Effects of PDGF Isoforms (AA, BB, AB and CC) on Migration and Proliferation of SaOS-2 Osteoblasts and on Migration of Human Osteoblasts. *Int J Biomed Sci* 5: 380-9.
- Colnot C. 2009. Skeletal cell fate decisions within periosteum and bone marrow during bone regeneration. *J Bone Miner Res* 24: 274-82.
- Colnot C, Lu C, Hu D, Helms JA. 2004. Distinguishing the contributions of the perichondrium, cartilage, and vascular endothelium to skeletal development. *Dev Biol* 269: 55-69.
- Colnot C, Thompson Z, Miclau T, et al. 2003. Altered fracture repair in the absence of MMP9. *Development* 130: 4123-4133.
- Crisan M, Yap S, Casteilla L, et al. 2008. A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell* 3: 301-313.
- Dai J, Rabie AB. 2007. VEGF: an essential mediator of both angiogenesis and endochondral ossification. *J Dent Res* 86: 937-950.
- Eleftheriou F, Yang X. 2011. Genetic mouse models for bone studies--strengths and limitations. *Bone* 49: 1242-54.
- Eshkar-Oren I, Viukov SV, Salameh S, et al. 2009. The forming limb skeleton serves as a signaling center for limb vasculature patterning via regulation of Vegf. *Development* 136: 1263-72.
- Felsenberg D, Silman AJ, Lunt M, et al. 2002. Incidence of vertebral fracture in europe: results from the European Prospective Osteoporosis Study (EPOS). *J Bone Miner Res* 17: 716-24.

- Ferguson CM, Mielau T, Hu D, et al. 1998. Common molecular pathways in skeletal morphogenesis and repair. *Ann NY Acad Sci* 857: 33-42.
- Fiedler J, Etzel N, Brenner RE. 2004. To go or not to go: Migration of human mesenchymal progenitor cells stimulated by isoforms of PDGF. *J Cell Biochem* 93: 990-998.
- Fiedler J, Leucht F, Waltenberger J, et al. 2005. VEGF-A and PlGF-1 stimulate chemotactic migration of human mesenchymal progenitor cells. *Biochem Biophys Res Commun.* 334: 561-568.
- Fiedler J, Roderer G, Gunther KP, Brenner RE. 2002. BMP-2, BMP-4, and PDGF-bb stimulate chemotactic migration of primary human mesenchymal progenitor cells. *J Cell Biochem* 87: 305-12.
- Franceschi RT. 2005. Biological approaches to bone regeneration by gene therapy. *J Dent Res* 84: 1093-103.
- Galotto M, Campanile G, Robino G, et al. 1994. Hypertrophic chondrocytes undergo further differentiation to osteoblast-like cells and participate in the initial bone formation in developing chick embryo. *J Bone Miner Res* 9: 1239-1249.
- Gamell C, Osses N, Bartrons R, et al. 2008. BMP2 induction of actin cytoskeleton reorganization and cell migration requires PI3-kinase and Cdc42 activity. *J Cell Sci* 121: 3960-70.
- Gentili C, Bianco P, Neri M, et al. 1993. Cell proliferation, extracellular matrix mineralization, and ovotransferrin transient expression during in vitro differentiation of chick hypertrophic chondrocytes into osteoblast-like cells. *J Cell Biol.* 122: 703-712.
- Gerber HP, Vu TH, Ryan AM, et al. 1999. VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. *Nat Med* 5: 623-628.
- Gerstenfeld LC, Cullinane DM, Barnes GL, et al. 2003. Fracture healing as a post-natal developmental process: molecular, spatial, and temporal aspects of its regulation. *J Cell Biochem* 88: 873-884.
- Godwin SL, Soltoff SP. 1997. Extracellular calcium and platelet-derived growth factor promote receptor-mediated chemotaxis in osteoblasts through different signaling pathways. *J Biol Chem* 272: 11307-12.
- Gomez-Barrena E, Rosset P, Muller I, et al. 2011. Bone regeneration: stem cell therapies and clinical studies in orthopaedics and traumatology. *J Cell Mol Med* 15: 1266-86.
- Grellier M, Bordenave L, Amedee J. 2009. Cell-to-cell communication between osteogenic and endothelial lineages: implications for tissue engineering. *Trends Biotechnol* 27: 562-71.

- Hah YS, Jun JS, Lee SG, et al. 2011. Vascular endothelial growth factor stimulates osteoblastic differentiation of cultured human periosteal-derived cells expressing vascular endothelial growth factor receptors. *Mol Biol Rep* 38: 1443-50.
- Hartmann C. 2009. Transcriptional networks controlling skeletal development. *Curr Opin Genet Dev* 19: 437-43.
- Haupt LM, Murali S, Mun FK, et al. 2009. The heparan sulfate proteoglycan (HSPG) glypican-3 mediates commitment of MC3T3-E1 cells toward osteogenesis. *J Cell Physiol* 220: 780-91.
- Helms JA, Schneider RA. 2003. Cranial skeletal biology. *Nature* 423: 326-31.
- Hengartner NE, Fiedler J, Ignatius A, Brenner RE. 2013. IL-1beta inhibits human osteoblast migration. *Mol Med* 19: 36-42.
- Henriksen K, Neutzsky-Wulff AV, Bonewald LF, Karsdal MA. 2009. Local communication on and within bone controls bone remodeling. *Bone* 44: 1026-33.
- Hill TP, Spater D, Taketo MM, et al. 2005. Canonical Wnt/beta-catenin signaling prevents osteoblasts from differentiating into chondrocytes. *Dev Cell* 8: 727-738.
- Hilton MJ, Tu X, Long F. 2007. Tamoxifen-inducible gene deletion reveals a distinct cell type associated with trabecular bone, and direct regulation of PTHrP expression and chondrocyte morphology by Ihh in growth region cartilage. *Dev Biol* 308: 93-105.
- Hughes A, Kleine-Albers J, Helfrich MH, et al. 2012. A class III semaphorin (Sema3e) inhibits mouse osteoblast migration and decreases osteoclast formation in vitro. *Calcif Tissue Int* 90: 151-62.
- Hughes FJ, Aubin JE, Heersche JN. 1992. Differential chemotactic responses of different populations of fetal rat calvaria cells to platelet-derived growth factor and transforming growth factor beta. *Bone Mineral* 19: 63-74.
- Ichida M, Yui Y, Yoshioka K, et al. 2011. Changes in cell migration of mesenchymal cells during osteogenic differentiation. *FEBS Letters* 585: 4018-24.
- Jacobsen KA, Al Aql ZS, Wan C, et al. 2008. Bone formation during distraction osteogenesis is dependent on both VEGFR1 and VEGFR2 signaling. *J Bone Miner Res* 23: 596-609.
- Kalajzic Z, Li H, Wang LP, et al. 2008. Use of an alpha-smooth muscle actin GFP reporter to identify an osteoprogenitor population. *Bone* 43: 501-510.
- Keramaris NC, Calori GM, Nikolaou VS, et al. 2008. Fracture vascularity and bone healing: a systematic review of the role of VEGF. *Injury* 39 Suppl 2: S45-57.
- Khosla S, Westendorf JJ, Modder UI. 2010. Concise review: Insights from normal bone remodeling and stem cell-based therapies for bone repair. *Stem Cells* 28: 2124-8.

- Komori T. 2010. Regulation of bone development and extracellular matrix protein genes by RUNX2. *Cell Tissue Res* 339: 189-95.
- Kronenberg HM. 2003. Developmental regulation of the growth plate. *Nature* 423: 332-336.
- Kronenberg HM. 2007. The role of the perichondrium in fetal bone development. *Ann NY Acad Sci* 1116: 59-64.
- Kulawig R, Kruger JP, Klein O, et al. 2013. Identification of fibronectin as a major factor in human serum to recruit subchondral mesenchymal progenitor cells. *Int J Biochem Cell Biol* 45: 1410-8.
- Kumar SPonnazhagan S. 2012. Mobilization of bone marrow mesenchymal stem cells in vivo augments bone healing in a mouse model of segmental bone defect. *Bone* 50: 1012-8.
- Kumar S, Wan C, Ramaswamy G, et al. 2010. Mesenchymal stem cells expressing osteogenic and angiogenic factors synergistically enhance bone formation in a mouse model of segmental bone defect. *Mol Therapy* 18: 1026-34.
- Lafage-Proust MH, Prisby R, Roche B, Vico L. 2010. Bone vascularization and remodeling. *Joint Bone Spine* 77: 521-4.
- Lee DH, Park BJ, Lee MS, et al. 2006. Chemotactic migration of human mesenchymal stem cells and MC3T3-E1 osteoblast-like cells induced by COS-7 cell line expressing rhBMP-7. *Tissue Eng* 12: 1577-86.
- Li G, Cui Y, Mcil Murray L, et al. 2005. rhBMP-2, rhVEGF(165), rhPTN and thrombin-related peptide, TP508 induce chemotaxis of human osteoblasts and microvascular endothelial cells. *J Orthop Res* 23: 680-5.
- Lienau J, Schmidt-Bleek K, Peters A, et al. 2009. Differential regulation of blood vessel formation between standard and delayed bone healing. *J Orthop Res* 27: 1133-40.
- Lind M, Deleuran B, Thestrup-Pedersen K, et al. 1995a. Chemotaxis of human osteoblasts. Effects of osteotropic growth factors. *APMIS* 103: 140-6.
- Lind M, Deleuran B, Yssel H, et al. 1995b. IL-4 and IL-13, but not IL-10, are chemotactic factors for human osteoblasts. *Cytokine* 7: 78-82.
- Lind M, Eriksen EF, Bunger C. 1996. Bone morphogenetic protein-2 but not bone morphogenetic protein-4 and -6 stimulates chemotactic migration of human osteoblasts, human marrow osteoblasts, and U2-OS cells. *Bone* 18: 53-7.
- Liu Y, Berendsen AD, Jia S, et al. 2012. Intracellular VEGF regulates the balance between osteoblast and adipocyte differentiation. *J Clin Invest* 122: 3101-13.
- Long F. 2012. Building strong bones: molecular regulation of the osteoblast lineage. *Nat Rev Mol Cell Biol* 13: 27-38.

- Maes C. 2013. Role and regulation of vascularization processes in endochondral bones. *Calcif Tissue Int* 92: 307-23.
- Maes C, Araldi E, Haigh K, et al. 2012a. VEGF-independent cell-autonomous functions of HIF-1 α regulating oxygen consumption in fetal cartilage are critical for chondrocyte survival. *J Bone Miner Res* 27: 596-609.
- Maes C, Carmeliet G, Schipani E. 2012b. Hypoxia-driven pathways in bone development, regeneration and disease. *Nat Rev Rheumatol* 8: 358-366.
- Maes C, Carmeliet P, Moermans K, et al. 2002. Impaired angiogenesis and endochondral bone formation in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. *Mech Dev* 111: 61-73.
- Maes C, Coenegrachts L, Stockmans I, et al. 2006. Placental growth factor mediates mesenchymal cell development, cartilage turnover, and bone remodeling during fracture repair. *J Clin Invest* 116: 1230-1242.
- Maes C, Goossens S, Bartunkova S, et al. 2010a. Increased skeletal VEGF enhances beta-catenin activity and results in excessively ossified bones. *EMBO J* 29: 424-441.
- Maes C, Kobayashi T, Selig MK, et al. 2010c. Osteoblast precursors, but not mature osteoblasts, move into developing and fractured bones along with invading blood vessels. *Dev Cell* 19: 329-44.
- Maes C, Stockmans I, Moermans K, et al. 2004. Soluble VEGF isoforms are essential for establishing epiphyseal vascularization and regulating chondrocyte development and survival. *J Clin Invest*. 113: 188-99.
- Mayer H, Bertram H, Lindenmaier W, et al. 2005. Vascular endothelial growth factor (VEGF-A) expression in human mesenchymal stem cells: autocrine and paracrine role on osteoblastic and endothelial differentiation 1. *J Cell Biochem* 95: 827-839.
- Mayr-Wohlfart U, Waltenberger J, Hausser H, et al. 2002. Vascular endothelial growth factor stimulates chemotactic migration of primary human osteoblasts. *Bone* 30: 472-477.
- Mehrotra M, Krane SM, Walters K, Pilbeam C. 2004. Differential regulation of platelet-derived growth factor stimulated migration and proliferation in osteoblastic cells. *J Cell Biochem* 93: 741-52.
- Mendez-Ferrer S, Michurina TV, Ferraro F, et al. 2010. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* 466: 829-34.
- Midy V, Plouet J. 1994. Vasculotropin/vascular endothelial growth factor induces differentiation in cultured osteoblasts. *Biochem Biophys Res Commun* 199: 380-386.

- Nakasaki M, Yoshioka K, Miyamoto Y, et al. 2008. IGF-I secreted by osteoblasts acts as a potent chemotactic factor for osteoblasts. *Bone* 43: 869-79.
- Nakashima K, Zhou X, Kunkel G, et al. 2002. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* 108: 17-29.
- Nakashima T, Hayashi M, Fukunaga T, et al. 2011. Evidence for osteocyte regulation of bone homeostasis through RANKL expression. *Nat Med* 17: 1231-4.
- Negishi-Koga T, Shinohara M, Komatsu N, et al. 2011. Suppression of bone formation by osteoclastic expression of semaphorin 4D. *Nat Med* 17: 1473-80.
- Otsuru S, Tamai K, Yamazaki T, et al. 2008. Circulating bone marrow-derived osteoblast progenitor cells are recruited to the bone-forming site by the CXCR4/stromal cell-derived factor-1 pathway. *Stem Cells* 26: 223-34.
- Panagakos FS. 1993. Insulin-like growth factors-I and -II stimulate chemotaxis of osteoblasts isolated from fetal rat calvaria. *Biochimie* 75: 991-4.
- Panagakos FS. 1994. Transforming growth factor--alpha stimulates chemotaxis of osteoblasts and osteoblast-like cells in vitro. *Biochem Mol Biol Int* 33: 643-50.
- Peng H, Wright V, Usas A, et al. 2002. Synergistic enhancement of bone formation and healing by stem cell-expressed VEGF and bone morphogenetic protein-4. *J Clin Invest* 110: 751-9.
- Pfander D, Kobayashi T, Knight M, et al. 2004. Deletion of *Vhlh* in chondrocytes reduces cell proliferation and increases matrix deposition during growth plate development. *Development* 131: 2497-508.
- Pignolo RJ, Kassem M. 2011. Circulating osteogenic cells: implications for injury, repair, and regeneration. *J Bone Miner Res* 26: 1685-93.
- Provot S, Zinyk D, Gunes Y, et al. 2007. Hif-1alpha regulates differentiation of limb bud mesenchyme and joint development. *J Cell Biol*. 177: 451-464.
- Rachner TD, Khosla S, Hofbauer LC. 2011. Osteoporosis: now and the future. *Lancet* 377: 1276-87.
- Raheja LF, Genetos DC, Wong A, Yellowley CE. 2011. Hypoxic regulation of mesenchymal stem cell migration: the role of RhoA and HIF-1alpha. *Cell biology international* 35: 981-9.
- Raheja LF, Genetos DC, Yellowley CE. 2008. Hypoxic osteocytes recruit human MSCs through an OPN/CD44-mediated pathway. *Biochem Biophys Res Commun* 366: 1061-6.

- Rankin EB, Wu C, Khatri R, et al. 2012. The HIF Signaling Pathway in Osteoblasts Directly Modulates Erythropoiesis through the Production of EPO. *Cell* 149: 63-74.
- Riddle RC, Khatri R, Schipani EClemens TL. 2009. Role of hypoxia-inducible factor-1alpha in angiogenic-osteogenic coupling. *J Mol Med* 87: 583-90.
- Roach HI, Erenpreisa J. 1996. The phenotypic switch from chondrocytes to bone-forming cells involves asymmetric cell division and apoptosis. *Connect Tissue Res* 35: 85-91.
- Rodda SJ, McMahon AP. 2006. Distinct roles for Hedgehog and canonical Wnt signaling in specification, differentiation and maintenance of osteoblast progenitors. *Development* 133: 3231-3244.
- Sacchetti B, Funari A, Michienzi S, et al. 2007. Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell* 131: 324-336.
- Saito T, Fukai A, Mabuchi A, et al. 2010. Transcriptional regulation of endochondral ossification by HIF-2alpha during skeletal growth and osteoarthritis development. *Nat Med* 16: 678-86.
- Saller MM, Prall WC, Docheva D, et al. 2012. Increased stemness and migration of human mesenchymal stem cells in hypoxia is associated with altered integrin expression. *Biochem Biophys Res Commun* 423: 379-85.
- Sanchez-Fernandez MA, Gallois A, Riedl T, et al. 2008. Osteoclasts control osteoblast chemotaxis via PDGF-BB/PDGF receptor beta signaling. *PLoS ONE*. 3: e3537.
- Schindeler A, McDonald MM, Bokko P, Little DG. 2008. Bone remodeling during fracture repair: The cellular picture. *Semin Cell Dev Biol* 19: 459-66.
- Schipani E, Maes C, Carmeliet G, Semenza GL. 2009. Regulation of osteogenesis-angiogenesis coupling by HIFs and VEGF. *J Bone Miner Res* 24: 1347-1353.
- Schipani E, Ryan HE, Didrickson S, et al. 2001. Hypoxia in cartilage: HIF-1alpha is essential for chondrocyte growth arrest and survival. *Genes Dev*. 15: 2865-2876.
- Semenza GL. 2012. Hypoxia-inducible factors in physiology and medicine. *Cell* 148: 399-408.
- Shapiro IM, Adams CS, Freeman T, Srinivas V. 2005. Fate of the hypertrophic chondrocyte: microenvironmental perspectives on apoptosis and survival in the epiphyseal growth plate. *Birth Defects Res C Embryo Today* 75: 330-339.
- Shen X, Wan C, Ramaswamy G, et al. 2009. Prolyl hydroxylase inhibitors increase neoangiogenesis and callus formation following femur fracture in mice. *J Orthop Surg* 27: 1298-305.

- Shomento SH, Wan C, Cao X, et al. 2010. Hypoxia-inducible factors 1alpha and 2alpha exert both distinct and overlapping functions in long bone development. *J Cell Biochem* 109: 196-204.
- Shum L, Coleman CM, Hatakeyama Y, Tuan RS. 2003. Morphogenesis and dysmorphogenesis of the appendicular skeleton. *Birth Defects Res C Embryo Today* 69: 102-22.
- Sims NA, Gooi JH. 2008. Bone remodeling: Multiple cellular interactions required for coupling of bone formation and resorption. *Semin Cell Dev Biol* 19: 444-451.
- Song L, Liu M, Ono N, et al. 2012. Loss of wnt/beta-catenin signaling causes cell fate shift of preosteoblasts from osteoblasts to adipocytes. *J Bone Miner Res* 27: 2344-2358.
- Street J, Bao M, Deguzman L, et al. 2002. Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover. *Proc Natl Acad Sci USA* 99: 9656-9661.
- Ström. 2011. Osteoporosis: burden, health care provision and opportunities in the EU. *Arch Osteoporos* 6: 59-155.
- Sugiyama T, Kohara H, Noda M, Nagasawa T. 2006. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity* 25: 977-88.
- Tabin C, Wolpert L. 2007. Rethinking the proximodistal axis of the vertebrate limb in the molecular era. *Genes Dev*. 21: 1433-1442.
- Taguchi K, Ogawa R, Migita M, et al. 2005. The role of bone marrow-derived cells in bone fracture repair in a green fluorescent protein chimeric mouse model. *Biochem Biophys Res Commun* 331: 31-6.
- Tang W, Yang F, Li Y, et al. 2012. Transcriptional regulation of Vascular Endothelial Growth Factor (VEGF) by osteoblast-specific transcription factor Osterix (Osx) in osteoblasts. *The Journal of Biological Chemistry* 287: 1671-8.
- Tang Y, Wu X, Lei W, et al. 2009. TGF-beta1-induced migration of bone mesenchymal stem cells couples bone resorption with formation. *Nat Med* 15: 757-765.
- Tare RS, Babister JC, Kanczler J, Oreffo RO. 2008. Skeletal stem cells: phenotype, biology and environmental niches informing tissue regeneration. *Mol Cell Endo* 288: 11-21.
- Towers M, Wolpert L, Tickle C. 2012. Gradients of signalling in the developing limb. *Curr Opin Cell Biol* 24: 181-7.
- Trueta J, Morgan JD. 1960. The vascular contribution to osteogenesis. I. Studies by the injection method. *J Bone Joint Surg Br* 42-B: 97-109.

- Tsuji K, Bandyopadhyay A, Harfe BD, et al. 2006. BMP2 activity, although dispensable for bone formation, is required for the initiation of fracture healing. *Nat Genet* 38: 1424-1429.
- Tsukamoto T, Matsui T, Fukase M, Fujita T. 1991. Platelet-derived growth factor B chain homodimer enhances chemotaxis and DNA synthesis in normal osteoblast-like cells (MC3T3-E1). *Biochem Biophys Res Commun* 175: 745-51.
- Vertelov G, Kharazi L, Muralidhar MG, et al. 2013. High targeted migration of human mesenchymal stem cells grown in hypoxia is associated with enhanced activation of RhoA. *Stem Cell Res Therapy* 4: 5.
- Wan C, Gilbert SR, Wang Y, et al. 2008. Activation of the hypoxia-inducible factor-1alpha pathway accelerates bone regeneration. *Proc Natl Acad Sci USA* 105: 686-691.
- Wan C, Shao J, Gilbert SR, et al. 2010. Role of HIF-1alpha in skeletal development. *Ann N Y Acad Sci* 1192: 322-6.
- Wang Y, Wan C, Deng L, et al. 2007. The hypoxia-inducible factor alpha pathway couples angiogenesis to osteogenesis during skeletal development. *J Clin Invest* 117: 1616-1626.
- Weiss HE, Roberts SJ, Schrooten J, Luyten FP. 2012. A semi-autonomous model of endochondral ossification for developmental tissue engineering. *Tissue Eng Part A* 18: 1334-43.
- Wildemann B, Burkhardt N, Luebberstedt M, et al. 2007. Proliferating and differentiating effects of three different growth factors on pluripotent mesenchymal cells and osteoblast like cells. *J Orthop Surg Res* 2: 27.
- Yang X, Tare RS, Partridge KA, et al. 2003. Induction of human osteoprogenitor chemotaxis, proliferation, differentiation, and bone formation by osteoblast stimulating factor-1/pleiotrophin: osteoconductive biomimetic scaffolds for tissue engineering. *J Bone Miner Res* 18: 47-57.
- Yano S, Mentaverri R, Kanuparthi D, et al. 2005. Functional expression of beta-chemokine receptors in osteoblasts: role of regulated upon activation, normal T cell expressed and secreted (RANTES) in osteoblasts and regulation of its secretion by osteoblasts and osteoclasts. *Endocrinology* 146: 2324-35.
- Yla-Herttuala S, Rissanen TT, Vajanto I, Hartikainen J. 2007. Vascular endothelial growth factors: biology and current status of clinical applications in cardiovascular medicine. *J Am Coll Cardiol* 49: 1015-1026.

FIGURE LEGENDS

Figure 1. Mechanisms of bone development. Different skeletal elements develop through intramembranous or endochondral ossification as indicated on a skeletal preparation stained with alizarin red (bone) and alcian blue (cartilage).

Figure 2. Stepwise representation of osteoblast (OB) differentiation from mesenchymal stem cells (MSC) with indication of typical stage-dependent markers. Transgenic lines that drive Cre expression in these cells at subsequent stages of osteoblast lineage cell maturation are indicated, as is the approximated developmental time of their activation.

Figure 3. Osteoblast lineage cell recruitment into developing long bones. Schematic drawing of the middle portion of a developing endochondral bone. Osteoblast lineage cells differentiate in the perichondrium surrounding the avascular cartilage anlagen. As described in the text, immature osteoprogenitors (Osx/LacZ⁺ cells) invade the cartilage and reach the primary ossification center in association with blood vessels. Histological and EM images (reproduced from (Maes et al., 2010c)) illustrate the pericytic localization of the Osx/LacZ⁺ cells in developing bones. The osteoprogenitors, blood vessels, and osteoclasts are attracted into the hypertrophic cartilage by chemotactic signals such as VEGF. Cells that differentiated to mature osteoblasts while residing in the perichondrium (Col1/LacZ⁺ cells) do not move into the primary ossification center to generate trabecular bone, but instead form the cortical bone on the outside.

Figure 4. Subsequent stages of endochondral fracture healing. In the first stage (upper left quarter), the injury site undergoes hematoma formation and initiates inflammatory and angiogenic responses. A plethora of growth factors is released in the callus. In the next days (post-fracture day (PFD) 4), cells in the periosteum covering the cortices adjacent to the injury site strongly proliferate, resulting in a thickened periosteum containing numerous Osx-Cre:GFP⁺ osteoprogenitors (upper right). Mesenchymal progenitors that are recruited to the fracture site differentiate into chondrocytes (CH) or osteoblasts (OB). Regions of cartilage in the callus are gradually replaced with immature woven bone via endochondral ossification (EO) (lower left). This process is associated with cartilage neovascularization (around PFD 7). Penetration of the cartilaginous callus regions by PECAM-1⁺ blood vessels coincides with recruitment of Osx-Cre:GFP⁺ osteoprogenitors into this area. The woven bone is further remodeled into in the final phase of the healing process by the actions of osteoclasts and osteoblasts (lower right). Fluorescent images from histological preparations are reproduced from (Maes et al., 2010c).

Figure 1

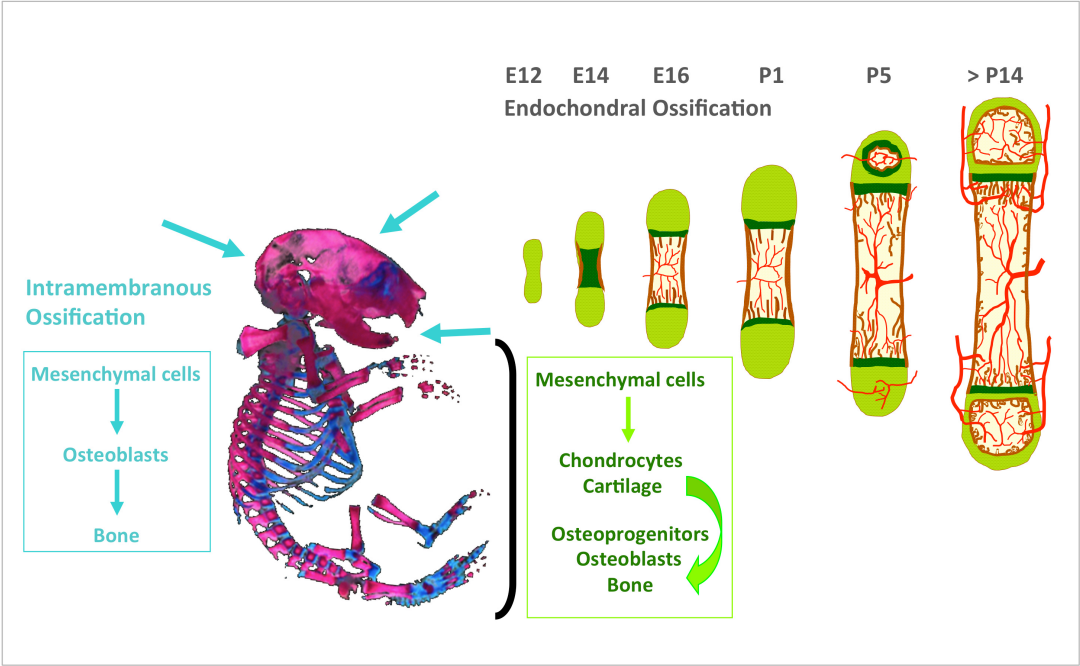


Figure 2

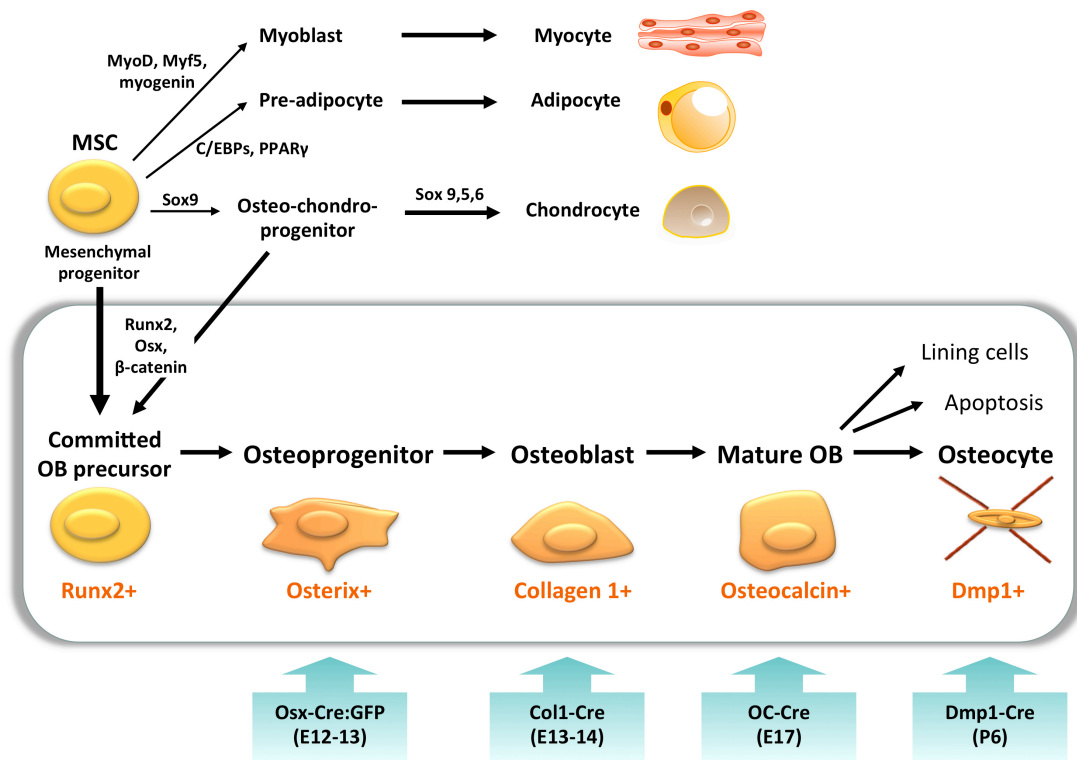


Figure 3

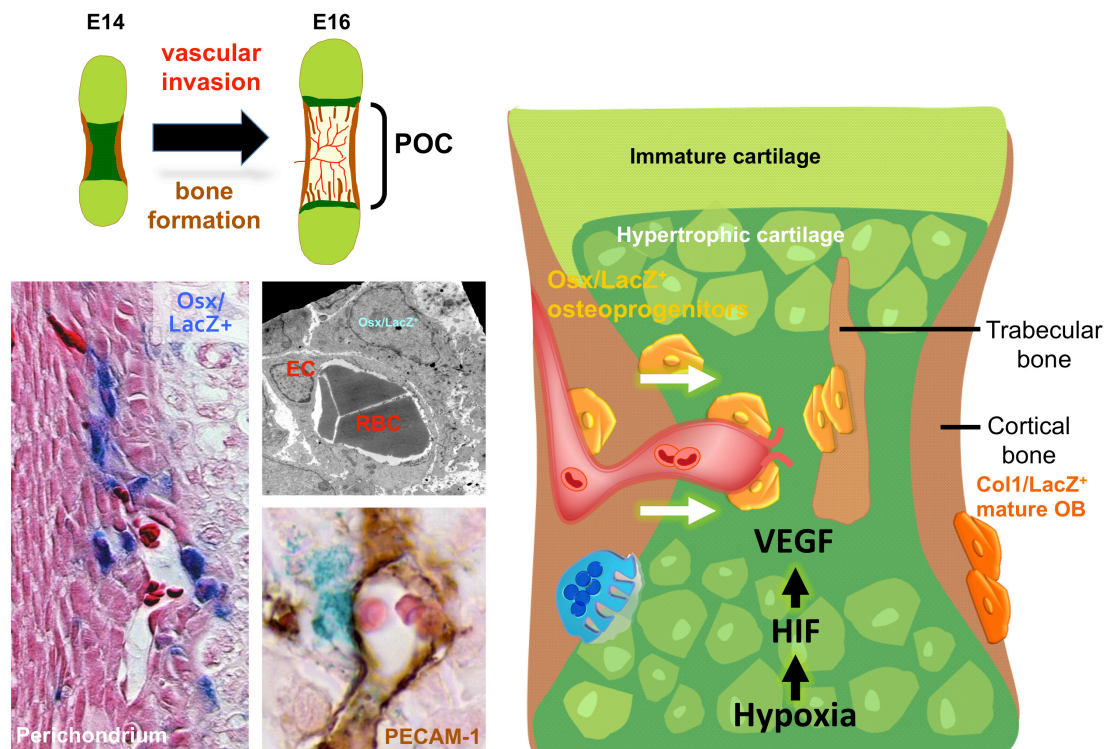


Figure 4

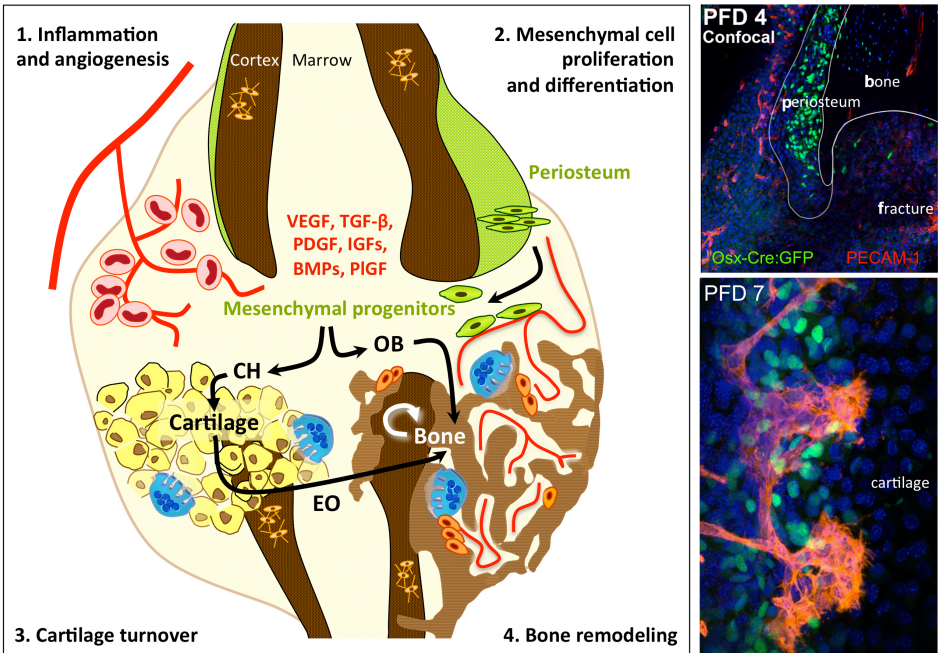


Table 1. Chemotactic signals for mesenchymal progenitor and osteoblastic cells, with a main focus on secreted growth factors

GF	Cells	Assay	Effect	Reference(s)
IGF				
IGF-1	MC3T3-E1 cell line; primary hOBs; mouse/rat calvaria cells	BCA, WHA	stimulation	(Hengartner et al., 2013, Lind et al., 1995a, Nakasaki et al., 2008, Panagakos, 1993)
IGF-2	primary hOBs; rat calvaria cells	BCA	stimulation	(Panagakos, 1993, Lind et al., 1995a)
PDGF				
PDGF-AA	SaOS-2 cell line; primary hOBs; hBMPCs	BCA	stimulation	(Colciago et al., 2009, Fiedler et al., 2004)
PDGF-BB	MC3T3-E1; SaOS-2; hBMPCs; primary hOBs; mouse/rat calvaria cells	BCA, WHA	stimulation	(Colciago et al., 2009, Fiedler et al., 2004, Fiedler et al., 2002, Godwin and Soltoff, 1997, Hughes et al., 1992, Mehrotra et al., 2004, Sanchez-Fernandez et al., 2008, Tsukamoto et al., 1991)
PDGF-AB	SaOS-2; hBMPCs; primary hOBs primary hOBs	BCA BCA	stimulation no effect	(Colciago et al., 2009, Fiedler et al., 2004) (Colciago et al., 2009)
PDGF-CC	SaOS-2; hBMPCs; primary hOBs	BCA	no effect	(Colciago et al., 2009)
TGF- β /BMP family				
TGF-α	primary hOBs; rat calvaria cells	BCA	stimulation	(Panagakos, 1994)
BMP-2	hBMPCs; primary hOBs; C2C12 cell line; bovine calvaria cells	BCA, DCA, WHA	stimulation	(Fiedler et al., 2002, Midy and Plouet, 1994, Gamell et al., 2008, Li et al., 2005, Mayr-Wohlhart et al., 2002)
BMP-4	U2-OS cell line; C2C12; primary hOBs	BCA	no effect	(Lind et al., 1996, Wildemann et al., 2007)
	hBMPCs	BCA	stimulation	(Fiedler et al., 2002)
BMP-6	U2-OS; hBMPCs; primary hOBs	BCA	no effect	(Lind et al., 1996)
	U2-OS; hBMPCs; primary hOBs	BCA	no effect	(Lind et al., 1996)
BMP-7	MC3T3-E1; hBMPCs	SCT	stimulation	(Lee et al., 2006)
VEGF family				
VEGF-A	SaOS-2; hBMPCs; primary hOBs; bovine calvaria cells	BCA, DCA	stimulation	(Fiedler et al., 2005, Hengartner et al., 2013, Li et al., 2005, Mayr-Wohlhart et al., 2002, Midy and Plouet, 1994)
VEGF-C	MC3T3-E1; hBMPCs	BCA	no effect	(Fiedler et al., 2005, Sanchez-Fernandez et al., 2008)
VEGF-E	primary hOBs	BCA	stimulation	(Mayr-Wohlhart et al., 2002)
	SaOS-2; hBMPCs	BCA	no effect	(Mayr-Wohlhart et al., 2002, Fiedler et al., 2005)
PIGF-1	SaOS-2; hBMPCs	BCA	stimulation	(Mayr-Wohlhart et al., 2002, Fiedler et al., 2005)
	primary hOBs	BCA	no effect	(Mayr-Wohlhart et al., 2002)

Interleukins			
IL-1β	primary hOBs hBMPCs	BCA BCA	inhibition no effect (Hengartner et al., 2013) (Hengartner et al., 2013)
IL-4	primary hOBs	BCA	stimulation (Lind et al., 1995b)
IL-10	primary hOBs	BCA	no effect (Lind et al., 1995b)
IL-13	primary hOBs	BCA	stimulation (Lind et al., 1995b)
Other			
bFGF	primary hOBs hBMPCs; bovine calvaria cells	BCA BCA	stimulation no effect (Lind et al., 1995a, Mayr-Wohlfart et al., 2002) (Midy and Plouet, 1994, Fiedler et al., 2002)
OSF-1	hBMPCs; primary hOBs	DCA, PSA	stimulation (Li et al., 2005, Yang et al., 2003)
Sema3a	mouse calvaria cells	WHA	inhibition (Hughes et al., 2012)
FN	hBMPCs	BCA	stimulation (Kulawig et al., 2013)
C5a	primary hOBs	BCA	stimulation (Hengartner et al., 2013)
CCL5	MC3T3-E1; rat calvaria cells	BCA	stimulation (Yano et al., 2005)
PTH	mouse calvaria cells	WHA	stimulation (Hughes et al., 2012)

Abbreviations: hBMPCs, human bone marrow progenitor cells; (h)OBs, (human) osteoblasts; BCA: Boyden chamber assay (transwell); WHA: wound healing assay (scratch test); DCA: Dunn chamber assay; PSA: patterned surface assay; SCT: single cell tracking. OSF-1, osteoblast-stimulating factor 1 (also known as pleiotrophin (PTN) or heparin-binding growth-associated molecule (HB-GAM)); FN, fibronectin; C5a, Complement component 5a; CCL5, Chemokine (C-C motif) ligand 5 (also known as Regulated on activation, normal T cell expressed and presumably secreted (RANTES)).

Table 2. Genetically modified mouse models of altered signaling of the osteo-angiogenic coupling factors VEGF and HIF during skeletal development, homeostasis and repair

Genetic model	Cre-driver	Phenotype	Reference(s)
Development			
VEGF			
VEGF cKO	Prx1-Cre	Absence of normal vascular branching in limb buds, leading to disturbed vascular morphogenesis at E10.5-E12.5	(Eshkar-Oren et al., 2009)
VEGF cKO	Col2a1-Cre	Chondrocyte cell death, delayed invasion of blood vessels into developing bones	Zelzer et al., 2004); (Maes et al., 2012a)
Rosa26-Vegf164 cTg	Col2a1-Cre	Hyper-vascularization and aberrant bone formation leading to skeletal malformations	(Maes et al., 2010b)
Vegf ^{120/120} KI	Exclusive expression of VEGF120 isoform (lack of VEGF164 and 188)	Impaired angiogenesis and endochondral ossification, inefficient cartilage resorption	(Maes et al., 2002)
Vegf ^{164/164} KI	Exclusive expression of VEGF164 isoform (lack of VEGF120 and 188)	Normal skeletal development and growth, normal vascularization and ossification	(Maes et al., 2004)
Vegf ^{188/188} KI	Exclusive expression of VEGF188 isoform (lack of VEGF120 and 164)	Chondrocyte cell death, disturbed development of the growth plate and secondary ossification center, dwarfism, knee joint dysplasia	(Maes et al., 2004)
HIF/VHL			
HIF-1 α cKO	Prx1-Cre	Limb shortening and malformation caused by impaired chondrogenic differentiation and formation of the cartilage anlagen and joints starting at E13.5	(Provot et al., 2007, Amarilio et al., 2007)
HIF-2 α cKO	Prx1-Cre	Slightly but transiently impaired differentiation to late hypertrophic chondrocytes causing shorter limbs at E17.5	(Araldi et al., 2011)
HIF-2 α het-KO		Impaired hypertrophic chondrocyte differentiation owing to Col10a1 suppression, resistance to osteoarthritis	(Saito et al., 2010)
HIF-1 α cKO	Col2a1-Cre	Massive cell death in central cartilaginous regions, skeletal malformations	(Schipani et al., 2001)
HIF-1 α cKO; Rosa-Vegf164 cTg (rescue)	Col2a1-Cre	Partial rescue of cell death in HIF-1 α deficient cartilage by VEGF164 overexpression	(Maes et al., 2012a)
HIF-1 α i-cKO (Tam at E15.5)	Col2a1-Cre-Ert	Hypocellularity in the growth plate at E17.5; impaired collagen hydroxylation and folding; ER stress and unfolded protein response activation, impaired ECM secretion	(Bentovim et al., 2012)
VHL cKO	Col2a1-Cre	Dwarfism, reduced chondrocyte proliferation, increased ECM production, hypocellularity and atypical large cells in resting zone	(Pfander et al., 2004)

Growth and Homeostasis			
tet(O)-VEGF164; Rosa26-rtTA i-cTg (Dox in adult)	Col2a1-Cre	Increased bone mass with excessive vascularization, bone marrow fibrosis and haematological abnormalities	(Maes et al., 2010b)
VEGF cKO	Osx-Cre:GFP	Reduced bone mass and increased bone marrow fat	(Liu et al., 2012)
VHL cKO	Osx-Cre:GFP	Increased EPO production in bone and expansion of erythroid lineage cells	(Rankin et al., 2012)
HIF-1 α /HIF-2 α d-cKO	Osx-Cre:GFP	Decreased bone volume and reduced erythroid progenitor population in bone marrow (causing no anemia physiologically)	(Rankin et al., 2012)
PHD1/2/3 triple-cKO	Osx-Cre:GFP	Dramatic increase of EPO levels in bone and of hematocrit value	(Rankin et al., 2012)
HIF-1 α cKO	Osteocalcin-Cre	Decreased bone volume, bone formation rate, blood vessel volume and number at 6 weeks. Impaired osteoblast proliferation.	(Shomento et al., 2010, Wang et al., 2007)
HIF-2 α cKO	Osteocalcin-Cre	No significant decrease in bone volume although blood vessel volume and number are significantly lower at 6 weeks of age	(Shomento et al., 2010, Wang et al., 2007)
VHL cKO	Osteocalcin-Cre	Extremely dense and heavy vascularized bones, no alteration in osteoblast proliferation and apoptosis	(Wang et al., 2007)
Repair and Regeneration			
PlGF KO		Impaired fracture healing characterized by reduced inflammatory and angiogenic responses, increased cartilage accumulation and reduced remodeling	(Maes et al., 2006)
HIF-1 α cKO	Osteocalcin-Cre	Less vascularization and deficient bone healing	(Wan et al., 2008)
VHL cKO	Osteocalcin-Cre	Increased vascularity and more bone formation during distraction osteogenesis	(Wan et al., 2008)

Abbreviations: (d)-cKO, (double) conditional knockout using Cre-LoxP recombination strategy; (i)-cTg, (inducible) conditional transgenic line; KI, knock-in model; (het)-KO, (heterozygous) ubiquitous knockout mouse; Tam, tamoxifen; Dox, doxycyclin.

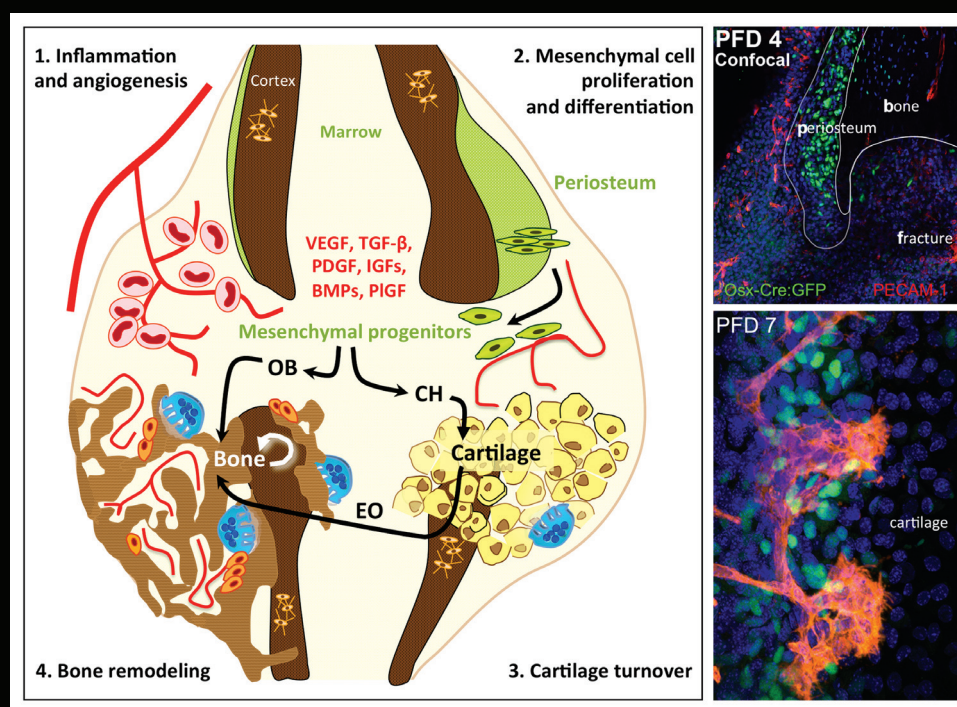
Volume 99, Number 3

September 2013

birth defects research

PART C:

EMBRYO TODAY: REVIEWS



Regeneration in Development

Rocky S. Tuan
Editor



ISSN 1542-975X

WILEY Blackwell